Analysis of Chk2, ATM, NBS1, three proteins involved in the cellular response to DNA damage

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ABSTRACT

In presence of damage to nuclear DNA, eukaryotic cells trigger a set of signal transduction pathways, which lead to the arrest of cell-cycle progression until repair has occurred, to the increase of DNA repair capacity or, in some cases to a controlled suicide (apoptosis). In human cells in presence of DNA damage Chk2 kinase protein is activated by ATM, the protein mutated in ataxia telangiectasia (AT), and targets several substrates involved in the global DNA damage response.

I demonstrated that Chk2 activation after DNA damage induction by γ-radiation treatment requires, besides ATM, Nbs1, the gene product involved in the Nijmegen Breakage Syndrome (NBS), a disorder that shares with AT chromosome fragility, radiosensitivity and radioresistant DNA synthesis. While in normal cells Chk2 undergoes a time-dependent increased phosphorylation and activation, in NBS cells these events are impaired. These defects can be complemented by the reintroduction of wild type copy of Nbs1 gene, but not by a deletion mutant encoding for a protein unable to form a complex with Mre11 and Rad50 in the nucleus. These results underline the main role of Nbs1 complex in ATM-Chk2 pathway in response to DNA damage and suggest that some defects in NBS and AT cells may result from defective Chk2 activation.

Since Nbs1 and ATM are involved in DNA breaks resolution, I investigated the lesion specificity of Chk2 response. Normal lymphoblastoid cells were exposed to different DNA damaging agents and proteins modifications and activation were evaluated. I demonstrated that ATM and Chk2 respond respectively to a low (<3) and a higher (>19) amount of double strand breaks per cell. Furthermore Chk2 activation was detectable only in response to double strand breaks occurrence but not to single strand breaks or lesions induced by UV or hydroxyurea. These data underline the specificity and the different sensitivity of DNA damage response components.

INTRODUCTION

During their life eukaryotic cells are under the constant attack of endogenous and exogenous stress sources. Particularly the biochemical structure of genomic DNA is highly sensible to a great variety of genotoxic agents. The maintenance of genomic stability is essential for a cell to survive and transmit with fidelity the DNA content to the daughter cell; therefore during evolution cells have developed a sophisticated mechanism, in order to prevent loss of genetic information, mutagenesis or cell death.

1. CAUSES OF DNA LESIONS OCCURENCE

Life exposes humans and all organisms to the constant presence of chemicals, radiation, and other sources that can damage DNA (Figure 1). Nucleotide changes in genome DNA, named mutations, while fuelling evolution can be detrimental to the organism and hence must be repaired to prevent uncontrolled cell growth and cancer, or cell death. In general, two are the main sources of genomic DNA lesions in a cell: endogenous and exogenous genotoxic agents.

i) Endogenous sources:

DNA damage occurs at high frequency in a normal cell life, due to products of the cellular metabolism (Barnes and Lindhal, 2004). These include reactive oxygen species (superoxide anions, hydroxyl radicals and hydrogen peroxide) derived from oxidative respiration and products of lipid peroxidation. Over 100 different oxidative modifications have been identified in DNA (Cadet *et al.*, 1997) and although cells have implemented a sophisticated antioxidant defense system, the rate of steady-state endogenous oxidative lesions in a mammalian cell has been estimated at 10^4-10^5 adducts per cell in mammals. Spontaneous disintegration of chemical bonds in DNA is also present under physiological

conditions, and base loss due to spontaneous hydrolysis of DNA glycosyl bonds is in the order of 10^4 events per day for a mammalian cell (Lindhal and Andersson, 1972).

Other spontaneous DNA lesions are alkylations, deaminations and replicational errors, this last occurring during normal DNA duplication, like base misincorporation errors and stalled replication forks (Loeb and Cheng, 1990).

ii) Exogenous sources: Environmental agents are the other major cause of DNA damage. The ultraviolet component of sunlight, ionizing radiation (IR) and numerous genotoxic chemicals affect daily eukaryotic cells (Figure 1).

UV light is a component of sunlight ranging in wavelength from 200 to 400 nanometers (nm). This range is usually divided into three groups: UVA (320-400 nm); UVB (290-320 nm); UVC (200-290 nm). Most of the UV in sunlight never reaches the earth; in fact, as a result of atmosphere and ozone layer nearly no UVC and very little UVB, the shortest and most energy-packed UV wavelengths, reach the earth's surface. UVB rays penetrate the epidermis and, reaching keratinocytes, could induce DNA damage in these cells. The major lethal lesions produced are pyrimidine dimers as a result of a covalent attachment between adjacent pyrimidines in one strand (Cadet *et al.,* 2005). These dimers, like bulky lesions from chemicals, block transcription and DNA replication and are lethal if unrepaired. They can stimulate mutation and chromosome rearrangement as well. UVA is already an important source of damage, because it comprises 95% of solar radiation, but in contrast to UVB the mutagenic potential of UVA is very limited and DNA damages could be induced by this wavelength only indirectly, because it is hardly absorbed by the DNA molecule.

Ionizing radiation was the first mutagenic agent described as its effects on genes were first reported in the 1920's. X- and gamma-rays are energetic enough to produce reactive ions (charged atoms or molecules) when they react with biological molecules; thus they are referred to as ionizing radiation. When a photon of radiation passes through a cell, the

energy deposited excites an electron, which may either damage the DNA directly or indirectly through the mediation of hydroxyl radicals from water which can diffuse to the DNA and cause damage (Breen and Murphy, 1995). The most frequent lesions produced by radiation are base damages but, in contrast to other genotoxic agents, also DNA breaks are produced (Khanna and Jackson, 2001). According to the lower energy necessary to break a single DNA chain, most of the damage which occurs after γ or x-irradiation of a cell leads to the generation of single strand DNA breaks (SSBs), which can be readily repaired using the opposite strand as a template. Occasionally, however, two breaks occur very close to each other on opposite strands, or directly opposite each other, which may result in a double strand break (DSB). Furthermore a part of SSBs and DSBs created by radiation are complex, because accompanied by additional base damage and therefore particularly difficult to be repaired. It has been estimated that in a mammalian cell radiation damage is comprised of approximately 40 DSBs, 1000 SSBs and 2700 base damages Gy^{-1} $cell^{-1}$ (Ward, 1994).

In human body the rapidly dividing cell types (blood cell-forming areas of bone marrow, gastrointestinal tract lining) are the most affected ones by ionizing radiation and the severity of the effects depends upon the received dose. The information about the dose-effects relation in humans is based upon accidental exposures of nuclear plant workers and victims of atomic bomb explosions, such as those in Hiroshima and Nagasaki:

sublethal dose (1-2.5Gy): nausea and vomiting early; 1-2 wk. latent period followed by malaise, anorexia, diarrhea, hair loss, recovery (latency due to time it takes hematopoietic or other damage to show up)

lethal dose (3.5-4.5Gy): nausea and vomiting early; 1 wk. latent period followed by above with more severe symptoms including internal bleeding; a 50% chance of death (LD50 : dose at which half of exposed individuals will die; ca. 4Gy for humans). Death is due to blood cell or gastrointestinal failure

supralethal dose (>6.5Gy): nausea and vomiting early, followed by shock, abdominal pain, diarrhea, fever and death within hours or days. Death is due to heart or central nervous system damage.

The studies about the effects of radiation are particularly relevant for human health, because cells are normally exposed to space radiation, but in addition human body could be exposed to IR during medical exam (i.e. tomography exam dose: 0.002-0.05Gy) and therapeutic treatments (i.e. therapeutic thyroid cancer treatment: dose to the thyroid 50-100Gy, dose to the whole body 0.05-0.15Gy).

Finally there are many chemical damaging agents capable to stress DNA structure. Some well-known examples are: nitrous acid (formed by digestion of nitrites used as preservatives in foods that causes deaminations), nitrosoguanidine, methyl methanesulfonate, ethyl methanesulfonate (all react with bases and add methyl or ethyl groups), psoralens (found in some vegetables and used in treatments of some skin conditions agents causing intra- and inter-strand crosslinks), peroxides and genotoxic compounds in cigarette smoke.

Collectively, nuclear DNA suffers a large amount of damage per cell per hour as summarized in Table 1.

2. THE DNA DAMAGE RESPONSE

Nuclear DNA damage in eukaryotic cells triggers a set of signal transduction pathways, which are collectively known as "the DNA damage response" (Figure 2). Many are the final effects of the DNA damage response: one well-known feature is the slowing or arrest of cell-cycle progression, as a result of what are termed DNA damage checkpoints, which delay key cell-cycle transitions until repair has occurred (Li and Zou, 2005). Other aspects of the DNA damage response include changes in chromatin structure

at the site of damage, and the transcriptional induction and posttranslational modification of various proteins involved in increasing DNA repair capacity (Rouse and Jackson, 2002). Finally, in presence of a large amount of DNA damage, a cell of a multicellular organism could also trigger a highly regulated suicide, a process named "apoptosis". In some cell types, instead of apoptosis, a stress induced premature senescence is in response to DNA damage (Wahl and Carr, 2001).

All these pathways are finely regulated and coordinated and could be rapidly turned on and off (Wang and Cho, 2004). Furthermore the same damaging lesion could activate different responses, if inferred in different moment of the cell cycle progression.

Considering the high rate of spontaneous damages present in normal cell, it's also conceivable that not every damage activates checkpoints. In some cases, the lesions may be relatively easy to repair and a quick reversion after initial detection may prevent initiation of a global DNA damage response.

The principal notions about the DNA damage response, with the exclusion of apoptosis, derive from studies in model organisms, particularly the budding yeast *Saccharomyces cerevisiae*, but it has been demonstrated that the DNA damage pathways are highly conserved during evolution.

2.1 DNA lesions detection

The first step that activated the DNA damage response is damage detection (Figure 2). The cell must be able to reveal many types of DNA lesions and very low levels of DNA damage anywhere in the genome. Studies in budding yeast have indicated that even a single persistent double strand break can be detected and, under some circumstances, can trigger a global DNA damage response (Sandell and Zakian, 1993). Therefore a single break, in a genome of 12 million base-pairs of DNA tightly packed into chromatin, can be efficiently detected. The rapidity and potency of the DNA damage response indicates the high sensitivity of signaling proteins involved and their capacity to greatly amplify the

initial stimulus (McGowan and Russell, 2004).

2.2 Cell cycle arrest

The normal eukaryotic cell cycle is separated into four different phases. The two key events of the cell division cycle are: S-phase, in which the entire genome is replicated, and mitosis (M-phase), where the chromosomes are segregated into two daughter cells, which separate at cytokinesis. Between the end of M and the beginning of S phase there is an interval, known as G1 (G=gap); a second interval, known as G2, separates the end of DNA synthesis from the beginning of the next M-phase. This linear description of the cell cycle, however, is not always obeyed and some differentiated cells exit the cell cycle into a quiescent phase known as G_0.

In proliferating cells, an adequate response to the presence of a significant amount of DNA damage requires more than repair of the DNA lesions. The threat posed by damage is particularly serious during the two major cell cycle events: DNA replication and mitosis. Replication can render mutations irreparable, while mitosis in presence of unrepaired strand breaks can lead to gross chromosomal aberrations in the daughter nucleus. In order to counter these threats to genomic integrity, eukaryotes employ mechanisms that arrest the cell cycle temporarily in order, to allow the time to repair the damage. These regulatory mechanisms, that do not allow the initiation of a new phase of the cell cycle until the damage is repaired, are named "checkpoints" (Figure 2). Specific checkpoints control G1-S, G2-M boundaries and different stages of the S phase. While G1 and G2 progressions could be completely arrested until cells have repaired the damage, S phase could only slow down to avoid that the structures present on replicating DNA stall and therefore collapse, creating a more severe situation.

2.3 DNA repair

All cells possess a large number of different types of repair system, each relatively specific for a certain type of DNA damage (for a review see Yu *et al.*, 1999; Figure 1).

Base modifications are corrected by Direct Reversal of Base Damage without the need to break the DNA backbone, while non-bulky damage to bases resulting from oxidation, methylation, deamination, or spontaneous loss of the DNA base and single strand breaks are corrected by Base Excision Repair (BER) through the cleavage of the DNA backbone.

Nucleotide Excision Repair (NER) is perhaps the most flexible of the DNA repair pathways, considering the diversity of DNA lesions it acts upon (mainly pyrimidine dimmers, but also bulky chemical adducts, DNA intrastrand crosslinks and some forms of oxidative damage). The common features of NER processed lesions are that they cause both a helical distortion of the DNA duplex and a modification of the DNA chemistry. NER system differs from BER because, even though in presence of only a single incorrect base, this nucleotide is removed along with many other adjacent ones, creating a large gap around the damage. The importance of NER pathway is underlined by the occurrence of two rare autosomal recessive disorders, characterized by various degree of photosensitivity (even to enhanced skin cancer predisposition) and associated with defects in some NER components: xeroderma pigmentosum (XP) and the Cockayne's syndrome (CS). Finally, Mismatch repair (MMR) corrects replication mistakes, such as base-base mismatches and insertion/deletion loops.

Among the many types of DNA damage that exist within the cell, double-strand breaks (DSBs) are probably the most dangerous ones, because free DNA ends are subjected to degradation, but also extremely reactive. Furthermore DSBs differ from most other types of DNA lesions and are intrinsically more difficult to eliminate, because they affect both strands of the DNA duplex and therefore they prevent the use of the complementary strand as a template for repair.

DSBs lesions should result from exogenous agents (ionizing radiation, chemotherapeutic drugs...), but also from endogenously generated reactive oxygen species or mechanical stress on the chromosomes. They can also be produced as secondary damages, when DNA replication forks encounter DNA single-strand breaks or other types of lesion (Vilenchik and Knudson, 2003) and they can also occur at the termini of chromosomes, due to defect in the metabolism of chromosome ends (telomeres). In addition, regulated DSBs are generated during meiotic recombination and during regulated rearrangements, such as V(D)J recombination and immunoglobulin class-switch.

Endogenous, regulated or spontaneous, and exogenous DSBs seem resolved by the same pathways. Erroneous rejoining of broken DNA DSBs are particularly mutagenic events, leading to the loss, amplification or translocation of chromosomal material. All these events can lead to tumorigenesis if stroke chromosomal region encoding a tumor suppressor or proto-oncogenes. In order to counteract the detrimental effects of DSBs, cells have evolved two distinct and complementary mechanisms to repair these lesions: homologous recombination (HR) and non-homologous end-joining (NHEJ).

HR-directed repair (Khanna and Jackson, 2001) corrects DSB defects in an error-free manner, using a mechanism that retrieves genetic information from a homologous, undamaged DNA duplex; for these characteristics is confined to act only in presence of a sister chromatid and therefore in S, G2 or M phase of the cell cycle. In contrast, for NHEJ an undamaged partner is not necessary and the two ends are ligated together, but sometimes this occurs after limited DNA degradation at the termini. Consequently, NHEJ is often prone to error, and small sequence deletions are usually introduced (Lieber *et al.*, 2003).

A so wide and specific set of responses allows eukaryotic cells to repair all kind of damages at very high rates and efficiencies as summarized in Table 2.

2.4 Apoptosis

In multicellular organisms, in presence of damage beyond repair, as an alternative and ultimate strategy for preventing the propagation of a mutated genome, cells could induce a programmed cell death, a mechanism named "apoptosis" (Norbury and Zhivotovsky, 2004).

Apoptosis is a highly conserved physiological form of cell suicide, which occurs normally in some cell types and is required in normal development and for the maintenance of tissue homeostasis (reviewed by Kerr *et al.*, (1994)). Programmed cell death is a highly regulated series of events, which can be distinguished from degenerative cell death or necrosis by morphological and biochemical criteria. During apoptosis the activation of a signal transduction cascade conducts to genomic DNA degradation: the nucleus shrinks and becomes pyknotic; fragments of nucleus and cytoplasm are pinched off as nuclear blebs, resulting in progressive shrinkage of cell size. These fragments are engulfed by macrophage, but they do not elicit the synthesis of inflammatory cytokines and therefore, in contrast to cell necrosis, do not activate an inflammatory response. Apoptosis is activated in response to different forms of physiological, pathogenic or cytotoxic stress. In response to DNA damage this machinery is essential, because it protects the organism at the expense of the individual cell.

2.5 Cell cycle arrest, senescence or suicide?

The p53 tumor suppressor protein plays a key role in the regulation of the cell cycle and cell death (Meek, 2004), other than in cell differentiation, DNA repair, senescence and angiogenesis. A Wild type (wt) p53 is essential for the prevention of cancer (Sharpless and DePinho, 2002), consistent with the high tumor incidence observed in p53 *null* mice and in the p53 heterozygosity, detectable in patients affected by the cancer prone Li-Fraumeni syndrome. It is estimated that approximately one half of human cancers contains a mutation in p53 and that in the majority of the remaining tumors the p53 signaling pathway

is inactivated by up-regulation of p53 inhibitors, such as Mdm2 and MdmX, or by down-regulation of p53 cooperators, such as ARF. This increased predisposition to tumor development in the absence of a functional p53 pathway is mainly due to the accumulation of genetic alterations and failure to eliminate these defective cells.

Wild type p53 is a labile protein with a short half-life, but accumulation and activation of the protein (Figure 3) can be triggered by a variety of stress signals, including DNA damage, hypoxia, nucleotide deprivation, viral infection, heat shock and mitogenic or oncogenic activation (Appella and Anderson, 2001). The specific activity of p53 is enhanced by post-translational modifications (Appella and Anderson, 2001) and by a wide spectrum of positive and negative regulators, ultimately leading to growth arrest and/or apoptosis (Figure 3). In normal non-stressed cells, the p53 protein level is very low and this is due to an autoregulatory feedback-loop mechanism in which the Mdm2 and MdmX proteins play a key role (Figure 3). Mdm2 interacts with p53 and this has two effects: first, the binding of Mdm2 to p53 may interfere with the interaction of p53 with the transcription machinery and second, Mdm2 can act as an ubiquitin E3 ligase on p53 and target it for degradation by the proteasome. MdmX, on the contrary, retains only the ability to interact and inhibit p53 transactivation activity, but could also be captured and degraded by Mdm2 (Marine and Jochemsen, 2004). If cells are subjected to a DNA-damaging agent, certain kinases such as DNA-PK, ATM, ATR or Chk2 may be activated and induce phosphorylation events on specific residues of p53, Mdm2 and MdmX in such a way that the effects of Mdm2 and MdmX on p53 may be impaired (Figure 3). The final effect of a p53 protein is accumulation and activation (Appella and Anderson, 2001). DNA damage inducing pro apoptotic transcriptional targets of p53 are Bax, Killer/DR5, p53RDL1, Bak, Puma, Noxa, PIG, Bid and, on the contrary p53 dependent cell cycle arrest inducers are $p21^{waf1}$, Rb, GADD45, p53R2 and Reprimo (for rewievs see El-Dehiry, 2003 and Fei and El-Dehiry, 2003).

The choice between growing arrest and apoptosis is determined mainly by characteristics and amount of the lesions. Specifically, for IR, the intensity of DNA damage appears to be an important determinant: lower doses of IR inducing reversible cell growth arrest (Kastan *et al.*, 1991) and higher doses of IR trigger apoptosis. This is obtained through the phosphorylation of different sets of p53 residues, according to the dose of damage. All these modifications ultimately differently affect the outcome of p53 response, because p53 binds to its responsive promoters with different affinity, due to sequence heterogeneity among the various responsive elements and the sequence specific DNA binding can be affected by changes in p53 conformation and by the conformation of cognate DNA sequences. An example of how different lesions could give rise to different cellular response is described by UV radiation and IR treatments. For instance, the osteosarcoma U2OS cells undergo apoptosis in response to UV, but undergo growth arrest upon IR. The accumulation of p53 in response to IR is rapid and transient, whereas in response to UV it is slow, intense and persists for long period. These differences can be explained, at least in part, by the different kinetics by which double strand breaks (DSB) versus bulky adducts are being repaired. The nucleotide excision repair (following UV) is slower than the DSB repair (following IR). Moreover, UV and IR trigger different signal transduction pathways, which ultimately lead to different pattern of post-translational modifications of p53, reflecting differential activation of upstream kinases. ATM is the critical upstream effector of p53 after IR, whereas p38 is essential for the UV-induced p53 activation, ATR and DNA-PK phosphorylate p53, following both signals.

The importance of DNA lesions amount and characteristics on the choice of outcome underlines that cell fate may be determined early when the damage is introduced, with p53 possibly integrating a plethora of signals. The signal imposed by DNA damage could be influenced by a variety of extracellular and intracellular conditions; an important role is recovered by the cell type (i.e. lymphocyte and fibroblast), by the presence of deregulated

expression of oncogene (i.e. in tumor cells) and by growth and survival factors (i.e. cytokines).

While the main cellular response to DNA damage is cell death or reversible cell-cycle arrest, in some circumstances, depending on the type of agent, the dosage administered and the type of cell treated, senescence could also occur (Wahl and Carr, 2001). This "premature senescence", both in the tissue culture and in the living mouse, can usually be prevented by p53 inactivation (Ongusaha *et al.*, 2003). It is not clear how cells choose between senescence and apoptosis upon DNA damage-induced p53 activation, but also in this case it has been suggested a role for p53 post-translational modifications (Webley *et al.*, 2000), interactions and the activation of different sets of transcriptional targets (Wahl and Carr, 2001). It is known that normal cells, in which all signaling pathways are intact, have a preference toward senescence when compared to transformed cells, and that senescence is usually induced by lower levels of DNA damage than those leading to apoptosis (Ben-Porath and Weinberg, 2005). The importance of the DNA damage response in senescence is also underlined by evidences that common proteins (i.e. γ-H2AX, Mdc1, Nbs1, ATM, Chk2, p53) are involved in the classical telomere-uncapping senescence (D'Adda di Fagagna *et al.*, 2003), but also after oncogene expression induced senescence (Serrano *et al.*, 1997).

2.6 DNA damage response defects: genomic instability and cancer

Left unrepaired, the myriad types of damage that can occur in genomic DNA could not only induce acute effects like transcription block or disturbed DNA metabolism and replication, but could also pose a serious threat to the faithful transmission of the genetic material. Over time, DNA accumulates changes that activate proto-oncogenes and inactivate tumor-suppressor genes. Therefore the genetic instability driving tumorigenesis is fuelled by DNA damage and by errors made by the DNA machinery (Lavin *et al.*, 2005), in addition to inherited faulty genome guardians that cause a mutator phenotype. Genome

instability is one of the main forces driving the onset and progression of carcinogenesis (Lavin *et al.*, 2005). Genetic degeneration is linked intimately with all aspects of maintenance of DNA integrity and gene function and is fuelled by the continuous erosion of the genome by environmental and endogenous genotoxic agents.

For some kind of tumors a "route to cancer" has been established; for example the number of genetic alterations required for a normal cell to progress to an invasive metastatic tumor varies from as many as 7, in the case of stomach cancer, to around 12 in the case of colorectal cancer (Renan, 1993). What emerges clearly is that a high number of mutations is necessary to create a tumor cell, while the spontaneous mutation rates in normal human cells cannot account for them. Therefore genomic instability, defined as all mechanisms that accelerate the accumulation of damage or modifications to DNA, has been proposed as an important process which contributes to the heterogeneity and progression of tumors. In particular the importance of cell cycle checkpoints in genomic stability (Hartwell and Kastan, 1994) is underlined by the observation that many inherited syndromes characterized by chromosomal instability and predisposition to malignancy (Kastan and Bartek, 2004), also possess defects in cell cycle regulation. As for instance all the major G1/S checkpoint transducers and effectors, with the exception of ATR, whose lack causes early embryonic lethality in mice and whose somatic defects might result in cell death, qualify as either tumor suppressors or proto-oncogenes, and their loss-of-function mutations or over-expression have been identified in many types of human malignancies. Hereditary mutations in at least ATM, Chk2, BRCA1, Mre11, Nbs1, p53, p16, and RB and other DNA damage involved gene are known to cause familial cancer and/or cancer prone clinical syndromes (Vessey *et al.*, 1999; Dasika *et al.*, 1999; Kastan and Bartek, 2004). The intimate involvement of cell-cycle checkpoints in molecular pathogenesis of cancer justifies the importance to explore this field in order to employ knowledge for diagnostic purposes and chemotherapy or radiotherapy treatment strategies.

3. ATAXIA TELANGIECTASIA, NIJMEGEN BREAKAGE SYNDROME AND ATAXIA TELANGIECTASIA LIKE SYNDROMES

3.1 Ataxia Telangiectasia

Ataxia Telangiectasia (AT) is an autosomal recessive disorder with a prevalence of 1/40,000 to 1/100,000 live births, characterized by early-onset cerebellar ataxia as its defining neurologic feature (for a review see McKinnon, 2004) (Table 3). Progressive cerebellar ataxia degeneration of Purkinje cells is almost always the presenting symptom and becomes apparent as early as the first year of life as increasing clumsiness and balance problems. By age 10 to 12, children with AT loose muscle control leading to wheelchair dependence by the second decade. As symptoms become progressively worse, speech becomes slurred and difficult. Telangiectases appear an average of two to four years after onset of the neurologic syndrome and are progressive. They are composed by dilated capillaries in the conjunctiva and later also on the ears, cheeks and nose. Other accompanying extra-neural features include elevated levels of serum alpha-fetoprotein, chromosome aberrations, immunodeficiency with recurrent sinopulmonary infection, cancer susceptibility, radiation hypersensitivity and endocrine disorders, such as insulin-resistant diabetes mellitus. During their lives, nearly 40% of AT patients will develop a malignancy (Morrell *et al.*, 1986) and about 85% of these malignancies will be leukemia or lymphoma. AT patients with cancer, treated with conventional doses of radiation therapy, result in devastating necrosis of normal tissues, demonstrating the high radiation sensitivity in these patients. The thymic hypoplasia with moderate cellular and humoral immunodeficiency is characterized by low levels of certain immunoglobulin classes (IgA, IgE, and IgG2), and T-cell deficiencies lead to frequent pulmonary infections in AT patients. The mean age of death is approximately in early or middle adolescence (about 17 years old), usually caused by bronchopulmonary infection, less frequently by malignancy, or the combination of both. In the absence of chronic bronchopulmonary disease and

lymphoreticular malignancy, AT is consistent with survival into the fifth or sixth decade of life.

Telomere shortening and fusions have been observed in peripheral blood lymphocytes of AT patients and in combination with the enhanced oxidative stress should also be responsible for progeroid features (gray hair or keratoses).

Mutations in the ATM gene are responsible for AT occurrence (Lavin and Khanna, 1999). At this moment ATM mutations have been described in more than 300 AT families: most of the patients are compound heterozygotes and, in many populations, there is a strong founder effect, but mutations are widely distributed and no hot spot has been identified. Currently, about 70% of the ATM mutations identified result in premature protein truncations, but there are also about 30% missense mutations and small in-frame deletions/insertions.

The carrier frequency for ATM is estimated at 1%, heterozygous carriers are normal neurologically, although they have in vitro radiosensitivity values that are intermediate between homozygotes and normals (Smilenov *et al.*, 2001). Several authors have reported that the incidence of cancer in AT heterozygotes is higher than the one in the general population, most notably breast cancer, but this is still controversial (Thompson *et al.*, 2005).

3.2 ATM

In order to identify the genetic defect responsible for ataxia telangiectasia multifaceted disorder, a genetic linkage analysis was performed mapping the AT-mutated (ATM) gene to chromosomal region 11q22-23, and finally the ATM gene, identified as a PI-3 kinase related protein (Savitsky *et al.*, 1995). The PI-3 kinase family of proteins (PIKKs) is characterized by a domain with motifs that are typical of the lipid kinase phosphatidylinositol 3-kinase (Figure 4) but, despite this sequence similarity, the catalytic domains of the PIKKs appear to transfer phosphate exclusively to protein, rather than lipid

substrates, and most of them possesses a specific serine/threonine kinase activity (for a review see Abraham, 2004). The mammalian members of this family, at present, include five protein kinases: ATM, ATR, ATX/SMG-1, mTOR/FRAP, DNA-PKcs and TRRAP, which is a component of histone acetyltransferase complexes. Four mammalian PI-3 kinases are known to be involved in the DNA-damage response: the DNA-dependent protein kinase (DNA-PK), ATM, ATR and ATX. ATM gene spans about 150 kilobases of genomic DNA, has 66 exons and is expressed in a wide range of tissues. The gene encodes a 350-kd (3056 amino acids) protein, in which the kinase domain represents less than 10% of the total ATM protein (Figure 4). Few other domains have been identified in ATM protein (Abraham *et al.*, 2001): a leucine zipper consensus sequence in the N terminus (until now there is no clear evidence that this motif is responsible for interaction of ATM with itself or with other proteins), and a proline-rich region that is a site for interaction with c-Abl, the non-receptor protein tyrosine kinase that has been implicated in processes of cell differentiation, cell division, cell adhesion, and stress response. ATM is localized mostly to the nucleus and has been shown to associate with DNA, with particular affinity for DNA ends (Abraham *et al.*, 2001), but is also found in cytoplasmic vesicles (Gately *et al.*, 1998). ATM kinase phosphorylates preferentially serine or threonine followed by glutamine (SQ/TQ), a sequence specificity shared with another PI-3 kinase, ATR. ATM and ATR both play a central role in the DNA damage response and appear to phosphorylate many of the same cellular substrates (for reviews see Tibbetts *et al.*, 2000; Zhou and Elledge, 2000; Khanna *et al.*, 2001; Rouse and Jackson, 2002). These kinases are activated by different forms of DNA damage and with different kinetics: ATM responds preferentially and rapidly to DNA double-strand breaks (DSBs) and therefore after treatments with ionizing radiation. On the contrary ATR functions following exposure to other forms of DNA damage, such as bulky lesions or stalled replication forks and therefore treatments with UV or hydroxyurea (O'Connell *et al.*, 2000; Khanna *et al.*, 2001) and only with a slow kinetics to radiation. In contrast to ATM, ATR is an essential gene required for cell proliferation

and in ATR null mice it causes early embryonic lethality (Brown and Baltimore, 2000); for this reason ATR is less studied and characterized. The yeast homologue of ATM is Tel1 in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, while ATR is the sequence and functional homologue of *S. pombe* Rad3 and *S. cerevisiae* Mec1. In both yeasts, Tel1 plays only a minor role in signaling the presence of DSBs, while Mec1- or Rad3-dependent pathways respond to all forms of DNA damage, underlining significant differences besides a general functional and structural conservation between yeasts and mammals cells.

The mechanisms for ATM activation in presence of DNA damage have been almost in part elucidated (Bakkenist and Kastan, 2003). In normally growing cells ATM is in a dimeric insoluble inactive form, but after the occurrence of DSBs, probably as a consequence of chromatin remodeling events, ATM autophosphorylates in Serine 1981 residue, becoming monomeric, soluble and active towards substrates (Figure 5). Up to now it's not clear if ATM is the primary and direct sensor of damage, or a mediator between the lesion and ATM is necessary to induce ATM autophosphorylation.

The number of ATM substrates and ATM–dependent events is wide and rapidly increasing. Many substrates are directly or indirectly involved in many aspects of the global DNA damage response (Figure 5) among them are: p53, Mdm2, MdmX, Chk2, 53BP1, Nbs1, Mre11, Brca1, H2AX, Mdc1, Smc1, FANCD2 and others, underlining the pivotal role of ATM in this field.

3.3 Nijmegen breakage syndrome

Nijmegen breakage syndrome is a rare inherited autosomal recessive disease (Table 3) originated in central Europe, in the Slavic population, and spread through a founder effect (Cerosaletti *et al.*, 1998; Varon *et al.*, 2000). More than 130 cases have been identified worldwide, including 68 in Poland and 26 in the Czech Republic. NBS shares a number of cellular features with AT, in particular a specific sensitivity to ionizing radiation

and radiomimetic chemicals. As a result, NBS has often been described as a variant of AT (Saar *et al.*, 1997). However, NBS patients lack many of the clinical hallmarks of AT such as progressive cerebellar ataxia, oculocutaneous telangiectasia and elevated serum alpha-fetoprotein levels. Clinically (Digweed and Sperling, 2004), NBS is characterized by microcephaly, a distinctive facial appearance described as "bird-like", growth retardation, immunodeficiency, frequent sino-pulmonary infections, chromosomal instability, radiation hypersensitivity and an increased incidence of malignancies, particularly lymphomas. Other manifestations are impaired sexual maturation in females, skeletal defects, urogenital malformations and skin pigmentation abnormalities. Intellectual abilities tend to decline over time and most children tested after the age of seven years have mild-to-moderate mental retardation. Immunodeficiency is usually characterized by defects in both cellular and humoral compartments. Due to defective immunity most, but not all NBS patients, show frequent respiratory, gastrointestinal and urinary infections. Constitutional karyotypes of NBS patients are generally normal, but cytogenetic aberrations are observed in 10-45% of metaphases from cultured T cells. As in AT, the vast majority of chromosome aberrations involve chromosomes 7 and 14. Typical and specific are inversions and translocations with breakpoints at the sites of immunoglobulin or T cell receptor genes. Malignancies occur at an elevated frequency and at an early age in NBS patients (mean age 9 years) and are almost exclusively lymphomas more often involving B-cells, in contrast with those found in AT. On the basis of available records, a 50-fold risk of early-onset cancer and a greater than 1000-fold risk of lymphoma are estimated for patients with NBS. Malignancy is the most common cause of death in infancy in patients with NBS; besides, a small number of patients died for bronchopneumonia. A polish patient 33 years aged is the longest survival known.

3.4 Nibrin (NBS1)

NBS disease is caused by defect in nibrin (NBS1) gene located in chromosome 8 (Varon *et al.*, 1998), coding for a 95KDa protein with 754 amino acids (Figure 4). Nibrin contains in the N-terminal a forkhead associated domain (amino acids 24-100) and a breast cancer domain (BRCT; amino acids 105-190) (Figure 4). Both domains are found in the various DNA damage responsive cell cycle checkpoint proteins and are involved in phosphoproteins interaction. Identified as the p95 subunit of the Mre11/Rad50/Nbs1 double-strand DNA break repair complex (Carney *et al.*, 1998; Trujillo *et al.*, 1998), nibrin is responsible for the nuclear localization and for the redistribution of the complex in foci (Tauchi *et al.*, 2001; Desai-Mehta *et al.*, 2001), associated with DSBs (Figure 6). Furthermore Nbs1 is essential for the nucleolytic activities of this complex, which is an essential step in NHEJ repair of DSBs. Nbs1 regulates the activities of the complex and is targeted by signaling pathways that initiate DNA damage-induced checkpoint responses (Figure 6). The Mre11/Rad50/Nbs1 complex shows also a role in meiotic recombination (Borde *et al.*, 2004) and telomeres maintenance. In more than 90% of patients tested so far, the common 657del5 mutation of Slavic origin is present in homozygosity. This mutation leads to a premature stop codon with a truncation at amino acid 219. The truncated protein (26KDa) and a 70Kda protein, produced by internal translation initiation within the NBS1 mRNA, using an open reading frame generated by the 657del5 frameshift, are detectable in NBS patients (Maser *et al.*, 2001). These two forms of truncated protein demonstrate that the NBS1 657del5 mutation is a hypomorphic defect and this could explain why Nbs1-null mouse are unviable (Zhu *et al.*, 2001). The remaining patients have a heterozygous 657del5 deletion and a second unique mutation or a homozygous unique mutation, in any case leading to a hypomorphic defect of Nbs1 protein.

3.5 Ataxia telangiectasia like disease

The cases of two cousins with the same clinical and cellular features of ataxia telangiectasia, but with a milder clinical course, were reported some years ago (Stewart *et al.*, 1999). In these two patients and in two other previously described ones, no ATM mutations were identified, while mutations in the MRE11 gene were revealed; therefore the new disease was named ATLD, for AT-like disorder (Table 3). Among the four patients two are homozygotes for a nonsense mutation at codon 633, and two compound heterozygotes for a null mutation and a substitution of serine for asparagine at amino acid 117. Both the nonsense and missense mutated genes produce stable protein products that are able to associate with Rad50 and Nbs1, although the re-localization of the full complex at DSB sites is abnormal (Stewart *et al.*, 1999). Data from *mMre11* knockout mice indicate that these human mutations are most likely partially functional, because a *null* allele of *mMre11* is lethal during embryogenesis (Xiao and Weaver, 1997), so as null alleles for *mRad50* (Luo *et al.*, 1999). Two cases of ATLD were described in Italy (Delia *et al.*, 2004) and 10 in Saudi Arabia (Fernet *et al.*, 2005).

3.6 Mre11

Orthologues of the DNA double-strand break repair nuclease Mre11 and the ATPase Rad50 exist in all organisms. Mre11/Rad50 complex from bacteria is capable of tethering DNA ends and possesses a large spectrum of DNA nuclease, helicase, ATPase and annealing activities (Connelly and Leach, 2002). Mre11 and Rad50 mutations in mice showed a severe effect on embryonic viability, revealing the requirement for Mre11 complex functions in early embryogenesis (Xiao and Weaver, 1997; Luo *et al.*, 1999). As described above, in eukaryotes the Mre11/Rad50 complex, in some circumstances, contains a third component, named Xrs2 in yeast and Nbs1 in vertebrates (M/R/N complex). Sedimentation equilibrium analysis of the recombinant human complexes indicates that M/R/N form large multimeric assemblies of approximately 1.2 MDa (Lee *et*

al., 2003). The minimal essential region for the interaction of Mre11 with Nbs1 was described in the C-terminus of Mre11 protein (Figure 4; Tauchi *et al.*, 2001). The human M/R/N complex is involved in meiotic recombination, homologous recombination, telomeres maintenance, DNA replication surveillance and DNA damaged structure detection and repair (D'Amours and Jackson, 2002). Particularly in DSBs the complex seems to enhance breaks repair holding DNA ends together in order, to ensure that they are coordinately and correctly rejoined (Figure 6). The ability of Mre11 to bind DNA was confirmed by the presence in this protein of two DNA binding motifs (Figure 4). Furthermore the complex could process DNA ends to make them accessible to subsequent reactions and could trap other DNA molecules, when necessary (i.e. during HR).

4. CHK2 KINASE PROTEIN

As previously described, Chk2 demonstrates an important role as signal transducer and particularly as a crucial link between the ATM/ATR kinases and downstream checkpoint effectors.

4.1 Chk2 homologs

The first member of the "Chk2 family" identified was Rad53 in budding yeast *Saccharomyces cerevisiae* and was described as a kinase involved in many checkpoint responses. Homologues of Rad53 were subsequently revealed in the fission yeast *Schizosaccharomyces pombe*, named Cds1, and in higher eukaryotes, while the identification of the human homologue was reported by two laboratories in 1998/1999 (Matsuoka *et al.*, 1998; Chaturvedi *et al.*, 1999). The main structure of the Chk2 proteins is conserved in all eukaryotes and the degree of homology is high and in accordance with the degree of evolution. Human CHK2 shows 83% and 82% amino-acid identity with the rat and mouse kinases: respectively, 61% identity with the *Xenopus laevis* protein; 51% with

the zebrafish; 34% with *Drosophila melanogaster*; 32% with both *S. pombe* and the nematode *Caenorhabditis elegans*; 28% with *S. cerevisiae* Rad53. In particular Rad53 differs from Chk2 for the presence, at carboxy terminus, of a second forkhead-associated (FHA) domain. Beyond the structural conservation, also the general role as transducers of cell cycle checkpoint signals is maintained throughout the evolution, although slight but significant functional differences between the members of the Chk2 family emerge, for example in lesion specificity or for the role in meiosis (Higashitani *et al.*, 2000). On the other hand, the human *CHK2* gene complements the checkpoint defects in both budding and fission yeast strains that are deficient in its homolog, thus underscoring an overall functional conservation among the Chk2 family members.

4.2 Chk2 gene and protein structures

The human *CHK2* gene spans 50 kilobases of genomic DNA, containing 14 exons and encodes a kinase protein that is characterized by some evolutionarily conserved elements (Figure 4, Bartek and Lukas, 2001). At the amino-terminal is present a series of seven serine or threonine residues followed by glutamine (SQ or TQ motifs); these are known to be favorable sites for phosphorylation by upstream kinases as ATM/ATR kinases and their homologues (Figure 4). Among these residues Thr68 in humans seems to display an essential role in Chk2 activation after DNA damage (Melchionna *et al.*, 2000). Another important region in Chk2 protein is the Fork Head Associated Domain (FHA) located between amino-acid residues 115 and 175 (Figure 4). This domain was identified in some transcription factors and in a range of mainly nuclear proteins with different functions. FHA domains seem necessary to bind phosphorylated residues and create protein–protein interaction (Durocher and Jackson, 2002), therefore is appears a good candidate for dynamic interactions of Chk2 with its upstream regulators and/or downstream targets in cell-cycle-checkpoint signaling. Finally the kinase domain occupies a large region in the

carboxy-terminal of Chk2, with its highly conserved functional elements, including the activation loop.

4.3 Chk2 protein activation

In order to describe Chk2 activation in humans, one model was proposed that seems to well resume the data until now obtained in higher eukaryotes (Figure 7). The first step is carried out by the ATM kinase, in response to double-stranded DNA breaks. This initial wave of phosphorylation targets serines or threonines in the regulatory SQ/TQ-rich domain of human Chk2 and particularly the residue Thr68 (Matsuoka *et al.*, 2000; Ahn *et al.*, 2000; Figure 7). The phosphorylation at Thr68 is a prerequisite for the dimerization of two Chk2 that is mediated by the interaction of the phosphorylated SQ/TQ domain of one molecule and the FHA domain of the other Chk2 protein (Ahn *et al.*, 2002; Figure 7). The final activating step is attributable to an *in trans* autophosphorylation of Chk2 on residues Thr383 and Thr387 in the activation loop of the kinase domain (Schwarz *et al.*, 2003; Figure 7). The initial phosphorylation in the SQ/TQ-rich domain might also evoke a conformational change in Chk2 that exposes the activation loop of the kinase and thereby facilitates its full activation. For the autophosphorylation step the presence of an FHA domain is also essential, which could be involved not only in Chk2 dimerization, but might also mediate the interaction of Chk2 with other checkpoint regulators. This mechanism of activation for Chk2 clearly resumes the basic characteristic of the kinase cascades involved in DNA damage signaling: a strictly regulated amplification of the initial signal. In fact a Thr68 phosphorylated Chk2 could activate others unmodified Chk2 molecules, by targeting the sites in the kinase domain. T68 phosphorylation is also essential for the relocalization of Chk2 in structures that appear as spot on DNA in an immunofluorescence analysis with a phosphospecific antibody (Ward *et al.*, 2001).

A role in Chk2 activation has been described also for two proteins, NFBD1/Mdc1 (Peng and Chen, 2003) and 53BP1 (Wang *et al.*, 2002), that are rapidly recruited at sites of

DNA damage (evaluated through the formation of foci by immunofluorescence techniques) and that seems to affect Thr68 phosphorylation and Chk2 redistribution at damage sites, as described in some reports (Ward *et al.*, 2001).

4.4 Chk2 functions

After the activation Chk2 propagates the DNA damage signal along several pathways, which causes cell-cycle arrest in the G1, S and G2/M phases, activation of DNA repair and, in some cases, apoptotic cell death or premature senescence (Figure 8).

Chk2 seems to impose a G1/S arrest through different effectors. Particularly Ser20 in p53 (Shieh *et al.*, 2000), Ser394 in E2F-1 (Rogoff *et al.*, 2004) and Ser123 in Cdc25A (Falck *et al.*, 2001) were described as potential Chk2 targets (Figure 8). These phosphorylation events seem to induce respectively p53 accumulation (Hirao *et al.*, 2000; Chehab *et al.*, 2000), E2F-1 activation (Stevens *et al.*, 2003) and Cdc25A degradation (Falck *et al.*, 2001), all events that contribute to a G1-S and S (only demonstrated for Cdc25A) progression block. In other papers the role for Chk2 in p53 accumulation has been questioned (Ahn *et al.*, 2003) and the essentiality for Cdc25A degradation partially revised. Particularly, more than in p53 stabilization Chk2 seems involved in activation of p53 and in induction of specific p53-dependent transcriptional targets like $p21^{waf1}$ (Hirao *et al.*, 2000). Therefore the phosphorylation of Ser20, a residue in Mdm2 interaction domain of p53, by Chk2 seems improbable. Another possible explanation for the role of Chk2 on p53 modulation is suggested by the ability of Chk2 to phosphorylated MdmX (Chen *et al.*, 2005). Indeed, after DNA damage MdmX protein is phosphorylated by Chk2 on Ser342 and Ser367 residues, thus stimulating MdmX interaction with 14-3-3 protein. This binding induces MdmX nuclear translocation, ubiquitination and degradation in an Mdm2-dependent way. The reduced level of MdmX protein contributes to p53 accumulation and activation (Chen *et al.*, 2005; Stad *et al.*, 2001).

The role in Cdc25A degradation after DNA damage, which is important not only in G1/S but also in S phase checkpoints, was instead questioned by recent reports about new and more effective Chk1 dependent phosphorylation sites in Cdc25A, that could lead to protein degradation through the interaction with the βTrCP protein (Jin *et al.*, 2003).

Chk2 role in G2/M checkpoint seems exerted through the phosphorylation of Ser216 in Cdc25C (Figure 8), a site known to be involved in negative regulation of this protein (Peng *et al.*, 1997). The importance of this event is made unclear by the fact that the same site is a target of Chk1 and p38 kinases, which could be however induced principally by UV treatments, more than IR, and by the unclear role for Cdc25C in G2/M arrest (Chen *et al.*, 2001). It's important to note that the description of every biological activity for Chk2 in human cells is complicated by the partial overlap activity of the structurally distinct Chk1 kinase. The functions of Chk1 protein cooperate, overlap or alternate with those of the Chk2, depending on the organism and on the nature of the genotoxic insult.

Although many papers have been published underlining the importance of Chk2 in checkpoint arrest after DNA damage in human cells, by the production of $Chk2^{-/-}$ mice and mouse cell lines contrasting data were obtained. One paper (Hirao *et al.*, 2000) shows in $Chk2^{-/-}$ murine cell lines the presence of defects in G1/S checkpoint and in G2/M block sustainment, but not in S phase, while another one (Takai *et al.*, 2002) shows defects only in G1/S checkpoint sustainment, both concordant only for defects in p53-dependent transcriptional activity. These data, in addition to the massive defects in cell cycle progression and checkpoint detectable in $Chk1^{-/-}$ mice (Takai *et al.*, 2000), suggest the hypothesis that Chk1 has a main role in checkpoint, but also in unperturbed cell cycles, whereas Chk2 shows a complementary role in regulating cell cycle progression after certain types of DNA damage, particularly DSBs. However this model is derived basically from data obtained in mouse and it's until now not demonstrated in humans and was not confirmed by data obtained from other organisms (see for example *Drosophila* mutants).

Chk2 has been also involved in DSBs repair, a role carried out through the functional interaction of Chk2 with Brca1 (Figure 8). BRCA1 protein is phosphorylated by Chk2 on serine 988 after IR treatments, and mutation of this residue prevents the dispersion of BRCA1 from sub-nuclear foci, that is imposed by DNA damage (Lee *et al.*, 2000). Furthermore the same phosphorylation event seems essential to induce the homologous recombination promoting activity of Brca1 (Zhang *et al.*, 2004). This functional interaction could also in part explain the data obtained from epidemiological evidence, which implicated Chk2 and BRCA1 in the same breast cancer prevention pathway (Sullivan *et al.*, 2002). It's not possible to exclude that Chk2-Brca1 relation could also be involved in cell cycle arrest, since Brca1 has been already implicated in this field.

Chk2 involvement in apoptotic activation is suggested by many observations: the only clear defect detectable in $Chk2^{-/-}$ mice and mouse embryo fibroblasts (MEFs) is that they fail to undergo DNA damage-induced apoptosis (Hirao *et al.,* 2000), the S516A mutant (a Chk2 autophosphorylation site) is defective in ionizing radiation-induced apoptosis and *Drosophila* homolog shows a role in DNA damage induced apoptotic events (Peters *et al.*, 2002). The relevance of p53 in this Chk2 activity (Figure 8) is controversial and only recently was proposed an effect of Chk2 not in inducing p53 accumulation but in activating the latent population of p53 preexistent to the post IR accumulation (Jack *et al.*, 2004). However other studies also propose a role of Chk2 in apoptosis through a p53-independent pathway, until now not elucidated. A possible pathway could act through E2F-1 protein, a Chk2 phosphorylation target (Figure 8); E2F-1 could prompt apoptosis through different pathways, p53-dependent or independent, inducing transcription of p14/Arf, p73 and Apaf1 (Pediconi *et al.*, 2003; Hallstrom and Nevins, 2003). An alternative mechanism is the Chk2 dependent phosphorylation of the proapoptotic protein PML (Yang *et al.*, 2002; Bernardi and Pandolfi, 2003).

Chk2 was also found interacting with two members of the Polo like kinases, Plk1 and Plk3 with roles until now not elucidated (Tsvetkov *et al.*, 2005; Xie *et al.*, 2002; Figure 8).

Emerging evidences suggest a role for Chk2 and other members of the DNA damage pathways in senescence response to telomere shortening (D'Adda di Fagagna *et al.*, 2003; Gire *et al.*, 2004), but also in premature senescence after uncontrolled replication, due to activation of oncogene Ras (d'Adda di Fagagna, personal communication). In this case senescence could be considered as an irreversible arrest of cell cycle progression, due to activation of checkpoint pathways also in absence of a direct damage. These observations could also be linked to recent data, demonstrating that in precancerous lesion, in presence of oncogene expression and consequently uncontrolled replication, the DNA damage response is constitutively activated, and particularly Chk2 is phosphorylated in the regulatory site threonine 68 (Bartkova *et al.*, 2005; Chen *et al.*, 2005).

The role of Chk2 as tumor suppressor, suggested for the involvement in the global checkpoint response was emphasized by the identification of germline and somatic mutations of the CHK2 gene in human hereditary and sporadic tumours, respectively. Mutations of *CHK2* have been found associated with the Li-Fraumeni-like multicancer syndrome (Bell *et al.*, 1999; Vahteristo *et al.*, 2001), with familial breast cancer (McPherson *et al.*, 2004) and with sporadic tumours as myelodysplastic syndromes, osteosarcomas, vulval neoplasia, breast cancer, lymphoid malignancies and rarely in lung, ovarian tumors and urinary bladder (for a review see Bartek and Lukas, 2003).

5. IONIZING RADIATION AS A MODEL TO STUDY THE DNA DAMAGE RESPONSE

The reason for the use of IR as a model to elucidate mechanism of DNA damage response is that cells have very specific mechanisms for the detection of radiation injury and for dealing with its consequences, either by repairing the damage, or by committing suicide. One of the reasons why cells possess these specific mechanisms is that organisms have evolved and adapted to the presence of radiation, in the form of sunlight (ultraviolet

light), from radioactive elements in the earth, and from space. Nuclear DNA damage is the critical firing of IR-induced cell response, in fact although there are cellular mechanisms suggesting that the cell membrane is a target of death in some instances (Haimovitz Friedman *et al.*, 1997), early experiments demonstrated that damage to the DNA is more than 3000 times more effective than membrane damage in the killing of cells *in vitro* (Warters and Hofer, 1977).

6. BASIC SIGNAL TRANSDUCTION CASCADE IN CHECKPOINT ACTIVATION

Cell cycle checkpoints are regulatory pathways that govern the order and the timing of cell cycle transitions, to ensure the correct completion of one cellular event before to the starting of another one.

6.1 The G1/S checkpoint

In presence of the correct conditions, during G1 phase of the cell cycle, cells could take the decision to replicate DNA and divide (for a review see Harper and Brooks, 2005). This decision is taken at the so-called 'restriction point' in mid-to-late G1 and seems irreversible until the next G1 phase. The main events that trigger S phase initiation are Cdk2 accumulation and activation (Bartek and Lukas, 2001). During G1 the presences of Rb-E2F complexes suppress transcription of genes required for DNA synthesis and particularly Cdk2 (Figure 9). Growth factors lead to increase in cyclin D, Cdk4/6 and E2F levels, Cdk4/6 phosphorylated Rb leading to E2F release, E2F induce transcription of cyclin E, Cdk2, E2F and others S phase regulators (Figure 6). Finally Cdk2 is activated not only by cyclin E interaction, but also by Cdc25A phosphatase dephosphorylation of inhibitory sites (Figure 9).

When a DNA damage is occurred in G1 phase, a cell cycle arrest is imposed through a pathway that requires transcription, translation and/or protein stabilization of key checkpoint transducers and, central to this pathway, is the accumulation and activation of the p53 protein (for a review see Fei and El-Deiry, 2003), while the final effect is the inhibition of Cdk2 activity and accumulation. Under normal conditions, p53 is a highly unstable protein and its DNA binding capacity is low. Furthermore, in normally growing cells, p53 levels are kept low due to interaction with Mdm2, which targets p53 for nuclear export and proteasome-mediated degradation in the cytoplasm (Figure 3). Furthermore MdmX interacts directly with p53 and inhibits its transactivation activity (Marine and Jochemsen, 2004). Following IR damage, ATM activates downstream kinases, which in turn phosphorylates residue serine 20 (Ser20) of p53. The Ser20 phosphorylation of p53 blocks p53/Mdm2 interaction, resulting in p53 escape from degradation and consequently accumulation (Figure 3). The Ser20 phosphorylation has been attributed for a large time to the ATM downstream kinase Chk2, but this assumption was questioned by different authors (Ahn *et al.*, 2003; Jallepalli *et al.*, 2003). ATM is capable to control p53 stability also by directly phosphorylating the p53 negative regulator, Mdm2, on Ser395 (Maya *et al.*, 2001). This modification allows Mdm2/p53 interaction, but prevents p53 nuclear export from the cytoplasm at site of degradation. A direct ATM and ATR phosphorylation site in p53 protein is Ser15, an event that seems crucial in enhancing p53 transcriptional transactivation activity, probably increasing its DNA binding capacity. Ser15 is rapidly targeted by ATM after IR and only with a slow kinetics by ATR, while in response to UV or replication block only ATR seems involved. ATM and Chk2 act in synergy not only on p53, but also in MdmX phosphorylation, another level for p53 activation regulation. Following DSBs, MdmX is phosphorylated by ATM and Chk2, leading to nuclear retention and subsequent degradation of this protein (LeBron *et al.*, 2006). MdmX is an inhibitor of p53 transactivation, therefore this destabilization could contribute to p53 activation after DNA damage. Numerous other post-translational modifications, such as

sumoylation, acetylation, seems necessary to lead to a complete stabilization of the p53 protein and activation of its sequence-specific DNA binding. Activated p53 up-regulates a number of target genes, several of which are involved in the DNA damage response and, among them, there are effectors for cell cycle arrest (Mdm2, GADD45α, Reprimo, and $p21^{waf1}$, Figure 3). Particularly the accumulation of $p21^{waf1}$, a protein that forms inhibitory complexes with a class of cdk-cyclins, leads to the suppression of Cyclin E/Cdk2 kinase activity, thereby resulting in G1 arrest (Figure 9).

However in mammalian cells if the damage occurs in late G1, when S phase has been already programmed, the G1/S checkpoint shows a two-wave model of arrest. An initial, rapid, transient and p53-independent response is activated before the delayed, but more sustained, arrest imposed by the p53–$p21^{waf1}$ pathway (Figure 9). The block of progression within minutes after DNA damage should be obtained through a mechanism independent of transcription and protein synthesis, two processes that require time consuming. For G1-S a way for a rapid activation of cell cycle arrest is the induction of Cdc25A degradation (Figure 9). The abundance and activity of Cdc25A rapidly decreases, when mammalian cells are exposed to DNA damaging agents, in consequence of poli-ubiquitination and subsequent degradation of the Cdc25A protein by proteasome (for a review see Bartek and Lukas, 2001). This degradation results in persistent inhibitory phosphorylation of CDK2 on tyrosine 15, and consequent inhibition of cyclin E–CDK2 activity with the final result to block G1/S transition. The signal for Cdc25A ubiquitination after damages is obtained through the activation of different pathways and is started by phosphorylation of Cdc25A in several residues. One of these aminoacids is serine 123, a target of the ATR/Chk1 and ATM/Chk2 pathway (Figure 9).

Therefore despite the fact that the initial steps are common for the Cdc25A and p53 pathways, their impact on G1/S progression are separated in time, due to the dependence of the latter pathway on transcription and protein synthesis.

6.2 The S phase checkpoint

DNA-modifying agents cause lesions that must be repaired before the DNA is copied, in order to prevent the replication machinery pairing an incorrect base with the modified one. Other agents, such as hydroxyurea, have the potential to damage chromosomes by interfering directly with the progress of replication forks, leading to incomplete replication and subsequent chromosome breakage. Cells respond to such events encountered during chromosome replication, by slowing the rate of DNA synthesis with existing replication forks being stabilized, whilst unfired late replication origins are prevented from initiating new replication forks. The role of S phase checkpoint is particularly important, because although the G2 checkpoint should block any cells that have exited S phase with damaged DNA, during S phase cells have the opportunity to perform error-free repair via homologous recombination, due to the presence of sister chromatids. Differing from other checkpoint pathway that cause a cell cycle arrest, the intra-S checkpoint could only slow down the progression in the cycle, although this can persist for several hours.

In budding yeast this checkpoint is largely studied and recently many aspects of this process have been elucidated. Specific role in S phase delay, fork stabilization and recombinational DNA repair have been attributed to Mec1 (ATM homolog), Rad53 (Chk2 homolog) and other proteins (Longhese *et al.*, 2003). On the contrary this pathway is the least understood of the mammalian checkpoints. Cells derived from individuals affected with ataxia telangiectasia (AT) or Nijmegen breakage syndrome (NBS) fail to slow their rate of DNA replication following IR exposure: a phenomenon named radio-resistant DNA synthesis (RDS). These findings implicate the associated gene products (ATM and NBS1, respectively) in the S-phase checkpoint pathway. Furthermore different proteins are activated during the S phase according to the damage inferred: IR could induce DNA breaks that induce multiple ATM-dependent cascades that slows DNA synthesis and

blocks new replication origin firing, while UV lesions fork arrest and replication errors could induce ATR/Chk1 and ATR/Brca1 responses leading to origin firing delay.

ATM has a leading role in almost two different signaling pathways, in response to IR damages during S phase progression. The first involve ATM/Chk2/Cdk2 pathway, the same that is functional in G1 (Figure 9). This according to the fact that Cyclin A/ or Cyclin E/Cdk2 complexes are essential to start (particularly Cyclin E/Cdk2), but also to progress (particularly Cyclin A/Cdk2) in DNA replication (Jackman *et al.*, 2002) (Figure 9). Therefore Cd25A degradation could also induce an intra-S checkpoint pathway to have an effect on Cdk2 activity, particularly blocking CyclinA/Cdk2 role in Cdc45 recruitment, during replication complex loading on origin and the resulting blockade of *de novo* initiation of late origins (Figure 9). However it is important to underline that the importance of Chk2 phosphorylation in Cdc25A Ser123 has been partly revised, because other kinases, phosphorylation sites and interactors have been discovered (Busino *et al.*, 2003; Jin *et al.*, 2003). As for other checkpoints, the ATM/Chk2 pathway seems central in response to IR, while ATR/Chk1 pathway has a major role after UV or HU treatments.

The second branch of the IR-induced S-phase checkpoint pathway is dependent, other than ATM, also on Nbs1 protein (Yazdi *et al.*, 2002). Upon IR damage, ATM phosphorylates a number of downstream substrates including NBS1 (at multiple sites including Ser343), BRCA1 (at multiple sites including Ser1387) and SMC1 (at Ser957 and Ser966), while Mdc1 protein recruits Mre11/Rad50/Nbs1 complex at site of DNA damage. Loss of any of these proteins (ATM, Nbs1, Brca1, Mdc1), or mutation in the indicated phosphorylation sites, results in defects of S-phase checkpoint activation. How this checkpoint is activated through this pathway and the relations between the involved proteins are until now unknown. A possible suggestion could arise from the fact that NBS1 and BRCA1 proteins are required for optimal phosphorylation of SMC1 upon IR and the ATM, NBS1, and BRCA1 proteins have all been shown to be part of a mega-dalton sized

protein complex named BASC (BRCA1-associated genome surveillance complex), that also include numerous other DNA repair and replication factors (Wang *et al.*, 2000).

6.3 The G2/M checkpoint

The G2 cell cycle checkpoint allows suspension of the cell cycle prior to chromosome segregation, a critical step in cell duplication (for a review see O'Connell *et al.*, 2000). Also in this case, as for G1/S checkpoint, in presence of a DNA damage an arrest could be induced through the induction of phosphatases activity on inhibitory site of cyclin dependent kinases. In G2/M the phosphatase is Cdc25C and the kinase is Cdc2 (Figure 10). Maintenance of the inhibitory phosphorylations on Cdc2 (on Thr14 and Tyr15) is one of the mechanisms for G2 checkpoint activation (Figure 10). Upon DNA damage, Chk1 and Chk2 kinases, activated by ATR- and ATM, respectively, phosphorylate Cdc25C on position Ser216 (Chaturvedi *et al.*, 1999). This phosphorylation creates a binding site for the 14-3-3 proteins that sequester Cdc25C in the cytoplasm, thereby preventing that this phosphatase activates Cdc2 through removal of the inhibitory phosphorylations (Dalal *et al.*, 1999, Figure 10). This results in the maintenance of the Cdc2/Cyclin B1 complex in its inactive state and blockage of entry into mitosis. A major role in this pathway is played by ATR and Chk1 also in response to IR other than, as usual, after treatments with UV. However, about the importance of this pathway contrasting data have been obtained (Lopez-Girona *et al.*, 2001).

Another possible target of ATM after DNA damage is Plk1 (van Vugt *et al.*, 2001), a kinase protein that shows activity on Cdc25C. Plk1 role in G2/M checkpoint was underlined by the inhibition of its activity after damage and by the partial abrogation of G2 arrest in cells with Plk1 mutants in the inhibitory sites. Plk1 was also found interacting with Chk2 (Tsvetkov *et al.*, 2005), underlining the role of both proteins in G2/M checkpoint.

P53 and $p21^{wafl}$ seems also to participate to G2/M checkpoint (Figure 10), particularly in some cell types. P53 has been described as a repressor of Cdc2 and cyclin B transcription and as a transcriptional inducer of Gadd45, a Cdc2 inhibitor acting as competitor of cyclin D1 (Taylor and Stark, 2001). $P21^{wafl}$ protein acts instead on G2/M progression, sustaining the block through a mechanism until now unclear, that seems to involve Cdc2 activation (Smits *et al.*, 2000).

Finally, in presence of an UV damage a G2 delay is also obtained through p38, a cytoplasmic kinase that, phosphorylating Cdc25B, abrogates the nuclear import of this phosphatase that acts on Cdc2 (Mikhailov *et al.*, 2005).

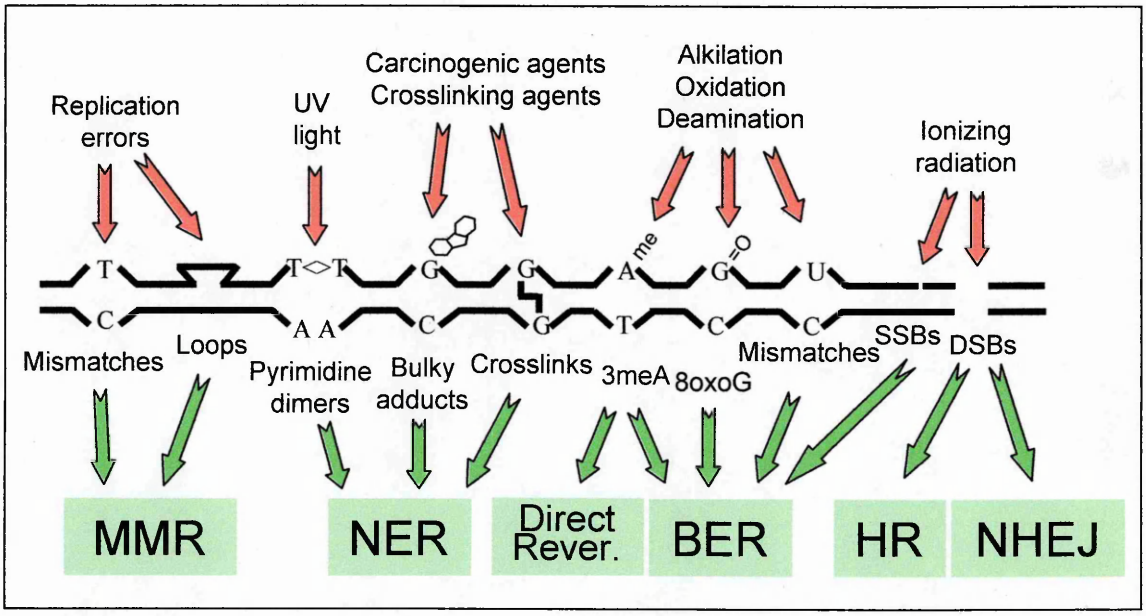

FIGURE 1: Schematic representation of the more relevant DNA damage sources, lesions and repairing systems.

Damage	Events per hour
Depurination	580
Depyrimidation	29
Deamination of Cytosine	8
Single-Stranded Breaks	2300
Single-Stranded breaks after depurination	580
Methylation of Guanine	130
Pyrimidine (thymine) dimmers in skin (noon day sun)	5×10^{4}
Single-stranded Breaks from Background Radiation	1×10^{-4}
Double-stranded Breaks from Background Radiation	4×10^{-6}

TABLE 1: DNA damaging events per hour per cell.

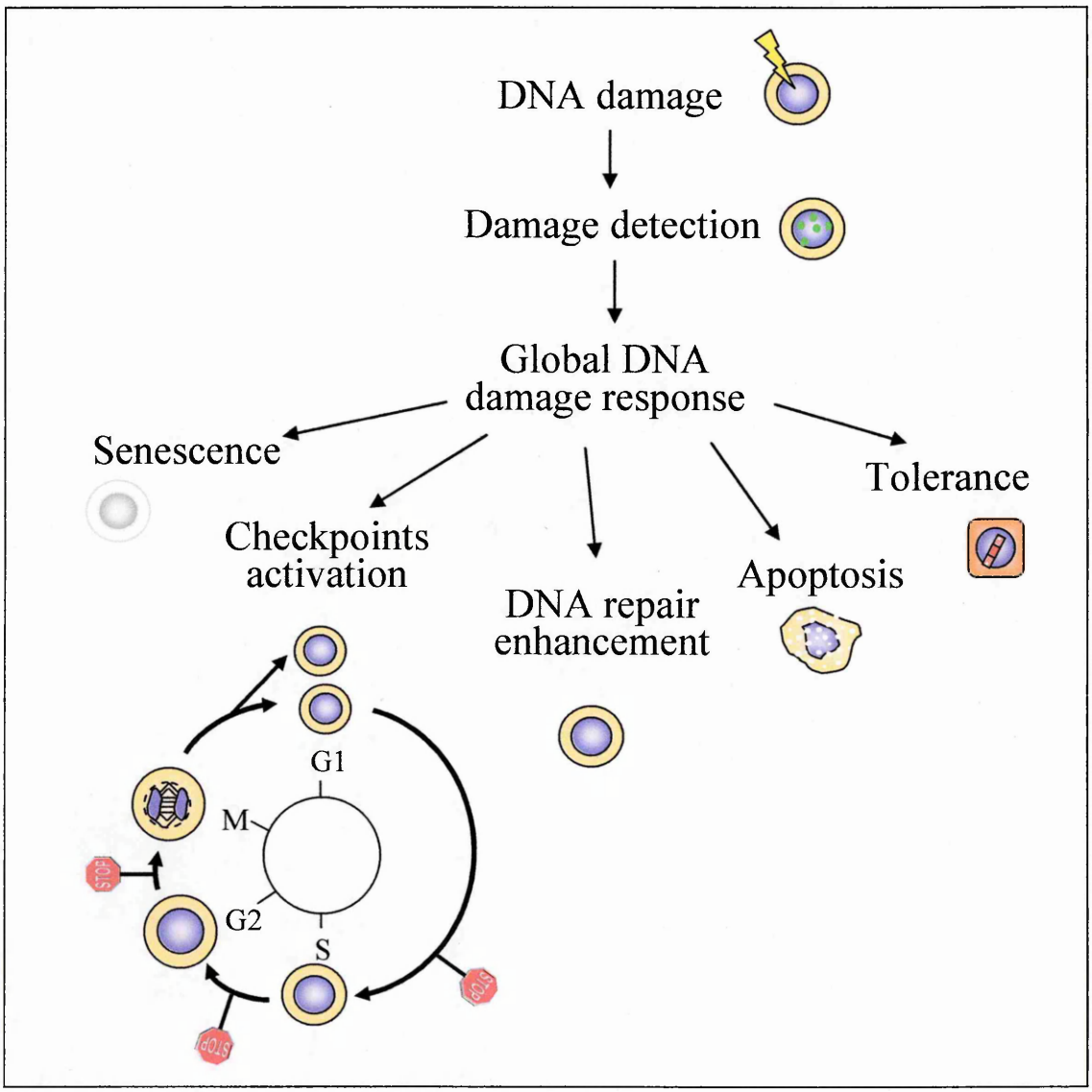

FIGURE 2: The global DNA damage response.

Damage	Repairs per hour
Single-stranded breaks	2×10^5
Pyrimidine dimers	5×10^4
Guanine methylation	10^4-10^5

TABLE 2: Maximum DNA repair Rates in a Human Cell.

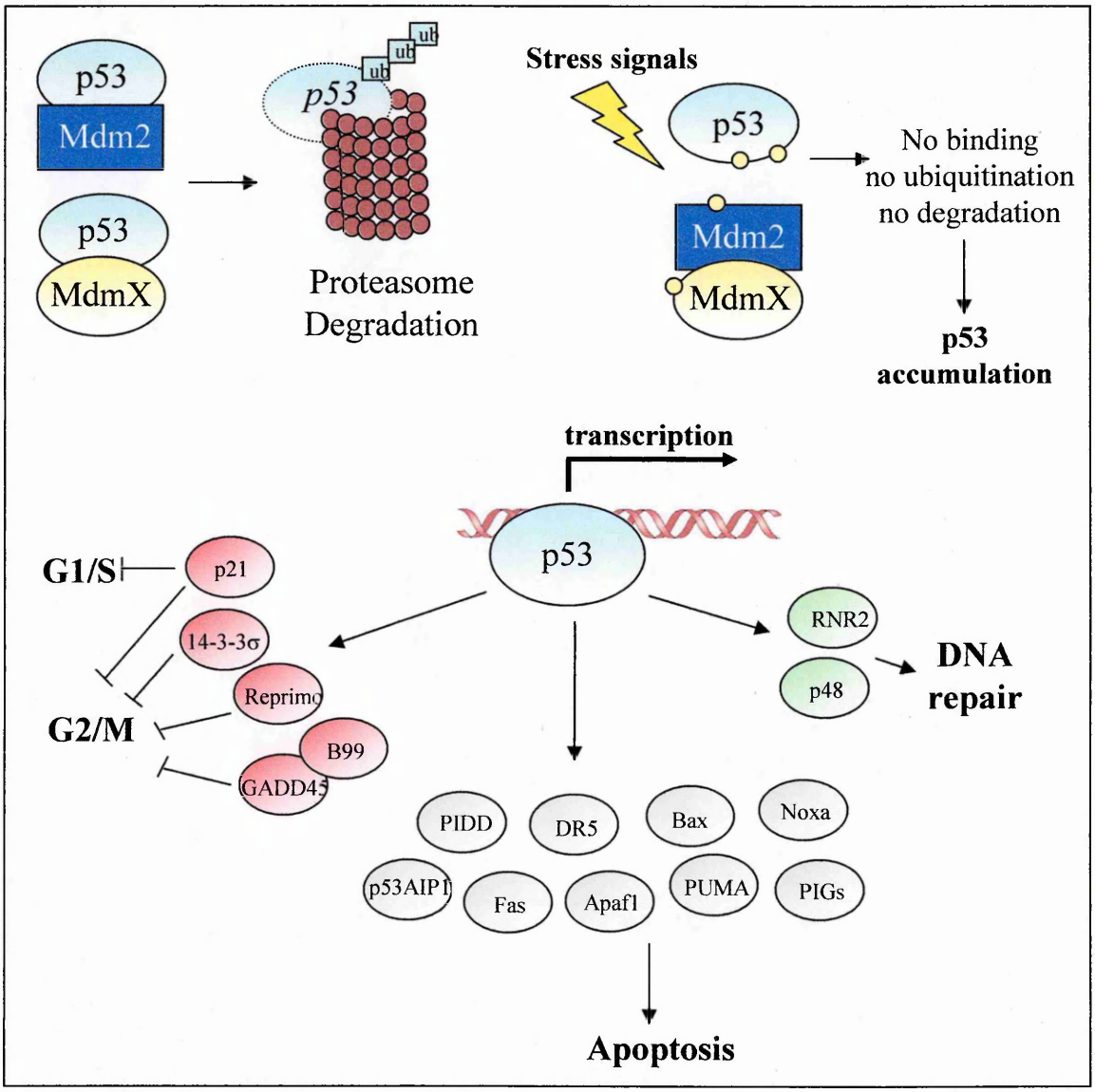

FIGURE 3: p53 activities in response to DNA damage. In absence of DNA damage (upper left) p53 protein is bound by Mdm2 or MdmX and conducted to degradation through the ubiquitination-proteasome pathway. When DNA damages occur p53, Mdm2 and MdmX proteins are modified principally by phosphorylation events, these leading to p53 accumulation (upper right) and transcriptional activity enhancement (lower). Some p53 targets and their role in DNA damage response are resumed.

Disease	Frequency	Neurological Features	Immunodef.	Chr. Instability	Cancer risk	Radiosens.	Other	Protein
Ataxia Telangiectasia	1/70000 live births	Cerebellar degeneration	cellular and humoral	+	100x cancer risk 70x leukemia 500x lymphoma	+	telangiectasia, frequent infections	ATM
Nijmegen Breakage Syndrome	~130 cases	Microcephaly Growth retardation	cellular and humoral	+	50x cancer risk 1000x lymphoma	+	frequent infections	Nbs1
Ataxia Telangiectasia Like Disease	6 cases	Cerebellar degeneration	-	+	-	+	no telangiectasia	Mre11

TABLE 3: Principal clinical and cellular features in Ataxia Telangiectasia, Nijmegen Breakage Syndrome and Ataxia Telangiectasia like Disease.

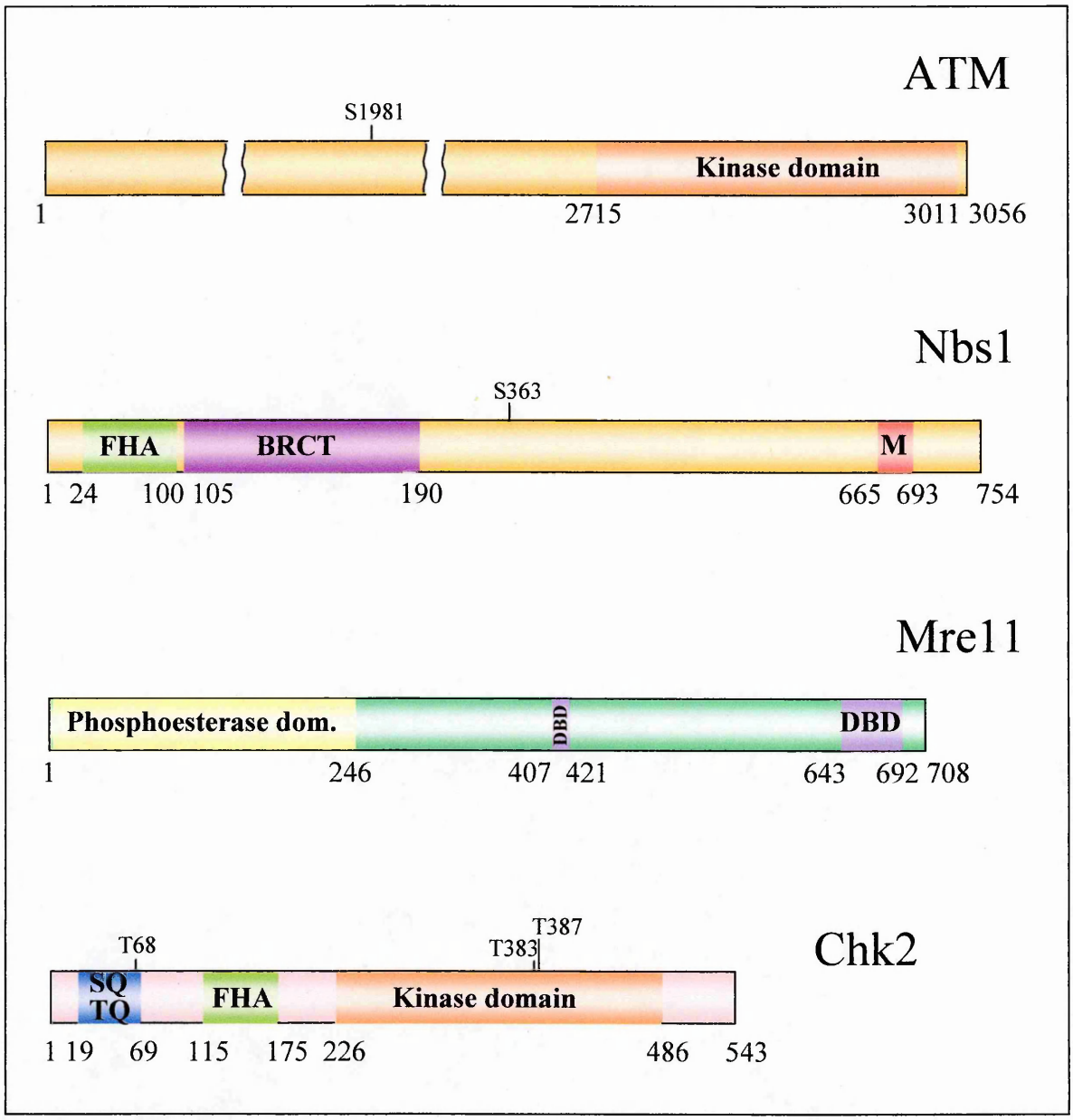

FIGURE 4: Schematic representation of ATM, Nbs1 and Chk2 proteins with known domains and phosphorylation sites. ATM is a large protein characterized by a C-terminal Kinase Domain and by a relevant autophosphorylation residue (S1981). In Nbs1 protein two highly conserved domains were described: a BRCT and an FHA domain are present in the N-terminus region. An Mre11 binding domain (M) is located at C-terminus, S363 is an ATM dependent phosphorylated residue. Mre11 is characterized by a phosphoesterase domain and two DNA binding domains (DBD) which are essential for its role in broken DNA ends tethering. Chk2 is a kinase protein with a highly conserved kinase domain, a Serine/Threonine rich domain in the N-terminus containing a large number of SQ and TQ sites which are preferentially substrate for ATM activity (T68 is the principal). A FHA domain is also present to bind phosphorylated proteins and dimerize. Dimerization leads to autophosphorylation in T383 and T387, two regulatory residues, producing a fully active kinase (see also Figure 7).

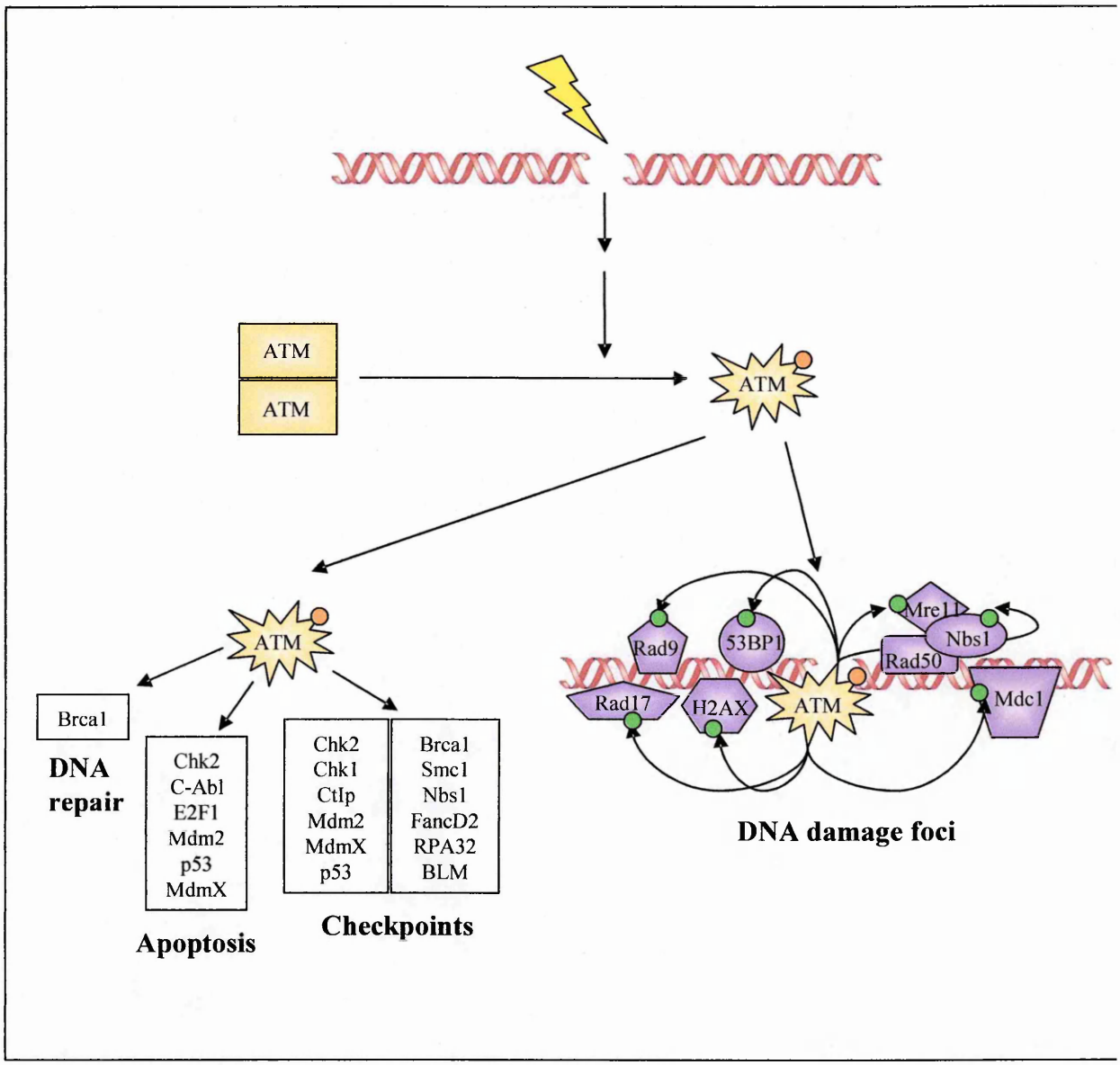

FIGURE 5: ATM activation and downstream activities. In presence of also a low number of double strand breaks and probably as a consequence of a chromatin rearrangement, ATM proteins, which normally is in a dimerized inactive state, after an autophosphorylation event in S1981 monomerize and change conformation becoming active. ATM kinase phosphorylated many downstream substrates, some of them are resumed in the left lower part of the figure. ATM is able to modify and induce the formation of protein agglomerates, named foci, that span many megabases around the lesion, and seems implicated in lesion protection, processing and repair and in checkpoint signal transduction.

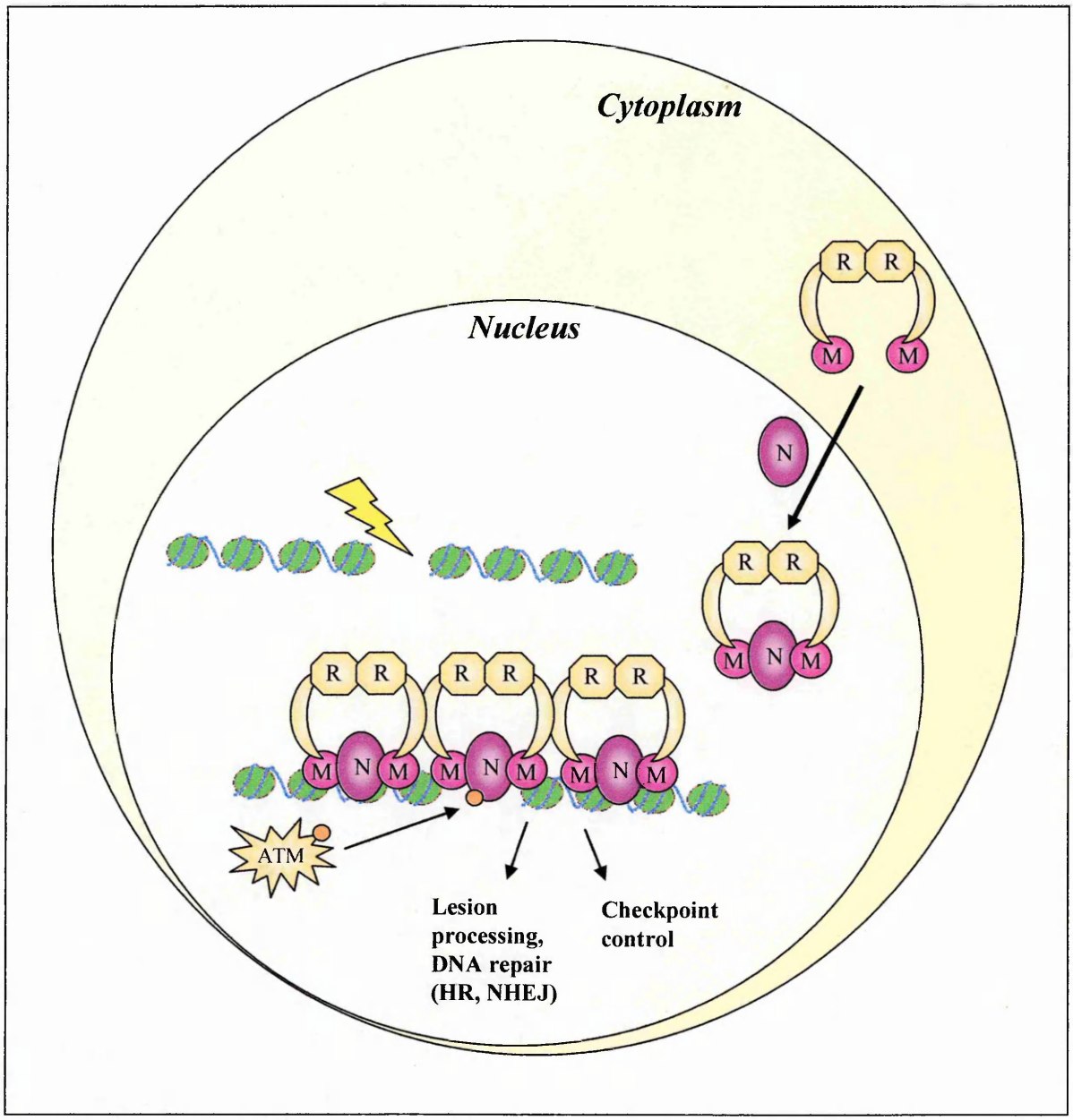

FIGURE 6: Schematic representation of Mre11, Rad50, Nbs1 complex formation and activity. Nbs1 (N) protein is essential to translocate the Mre11 (M) and Rad50 (R) complex. In absence of Nbs1the same complex is unstable and located in cytoplasm. In presence of a damage ATM phosphorylates Nbs1 in Ser343 leading to the formation of large agglomerates of many molecules of M/R/N complex around the damage (foci). M/R/N can tether DNA extremities in close proximity and with exo and endonuclease activity can process the lesion, enhancing recombination and stimulating DNA repair. The same complex shows a role in checkpoint regulation.

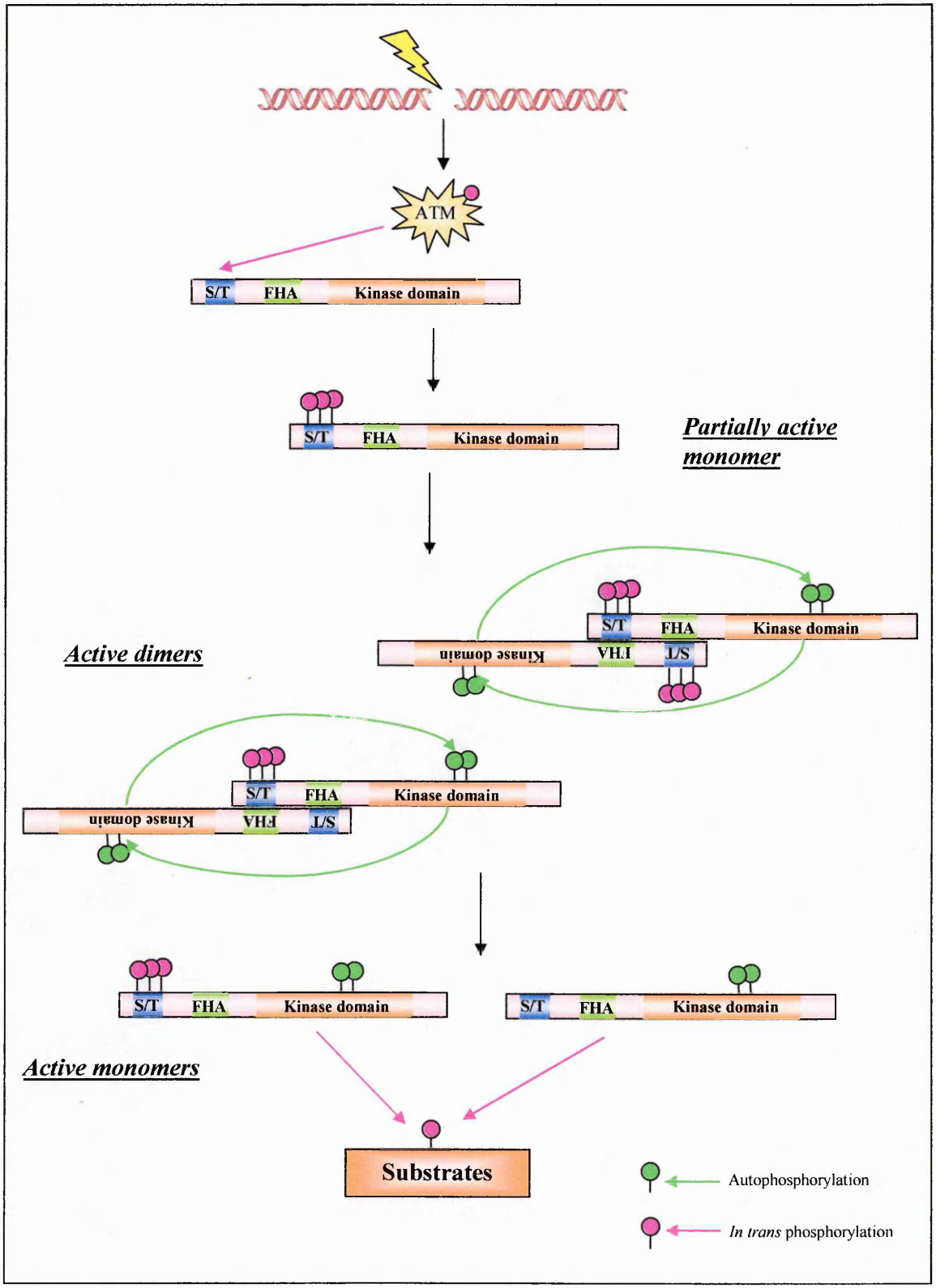

FIGURE 7: Schematic description of Chk2 activation events. When a DNA damage occurs ATM is activated and phosphorylates Chk2 in the TQ/SQ domain (S/T), leading to a partial induction of its kinase activity. The phosphorylated monomeric form of Chk2 dimerize through the interaction between S/T region and the FHA domain of another Chk2 molecule that could be either phosphorylated or not. Dimerization leads to *in trans* autophosphorylation and dimers breakage, with the final result of two active molecules.

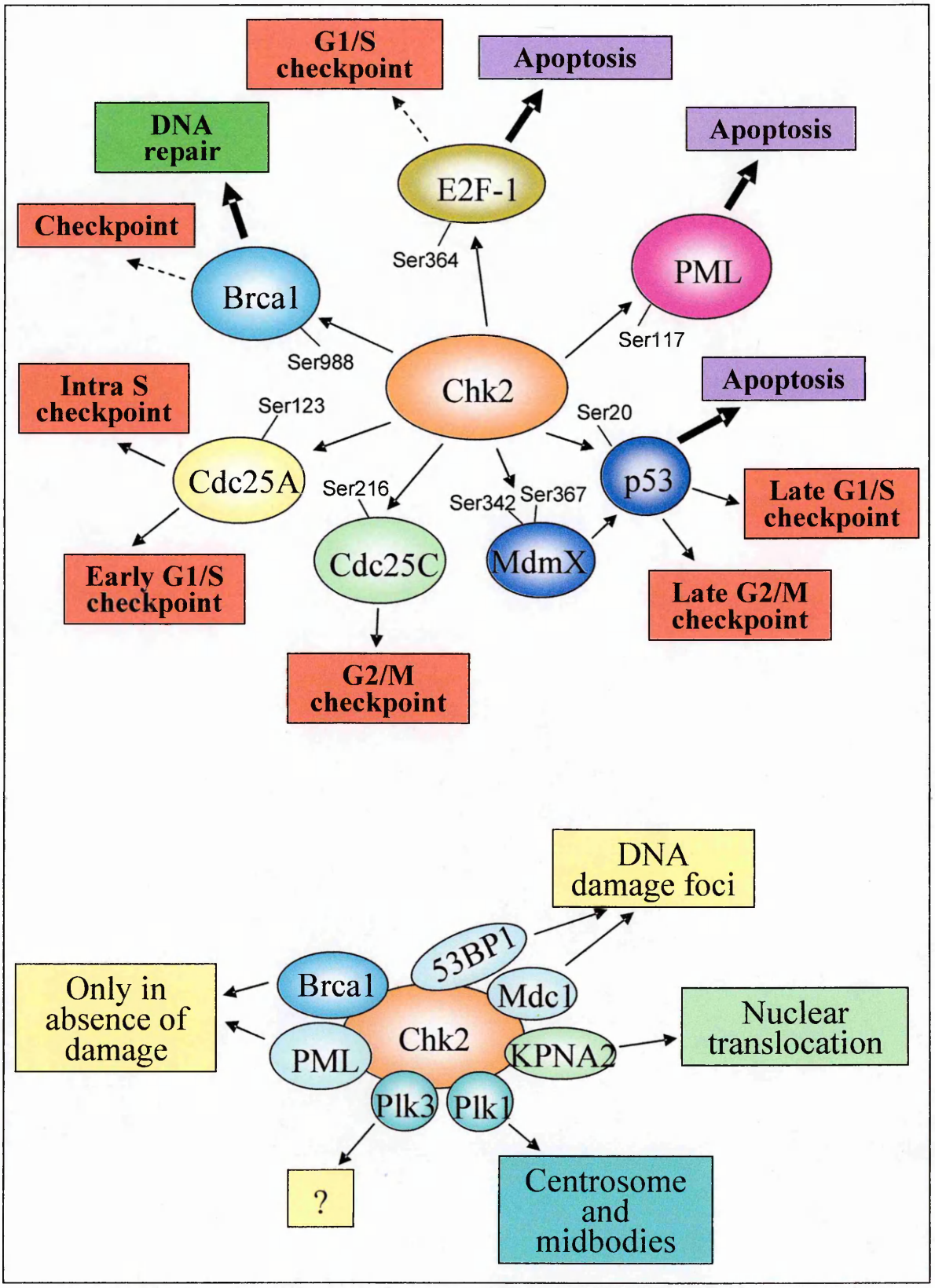

FIGURE 8: Chk2 substrates and interactors. In the upper part of the figure Chk2 substrates are summarized with the target residue and the role of these modifications in the global DNA damage response. In the lower part physical interactors are visualized, with specified, when known, the compartment, the condition or the role of the interaction.

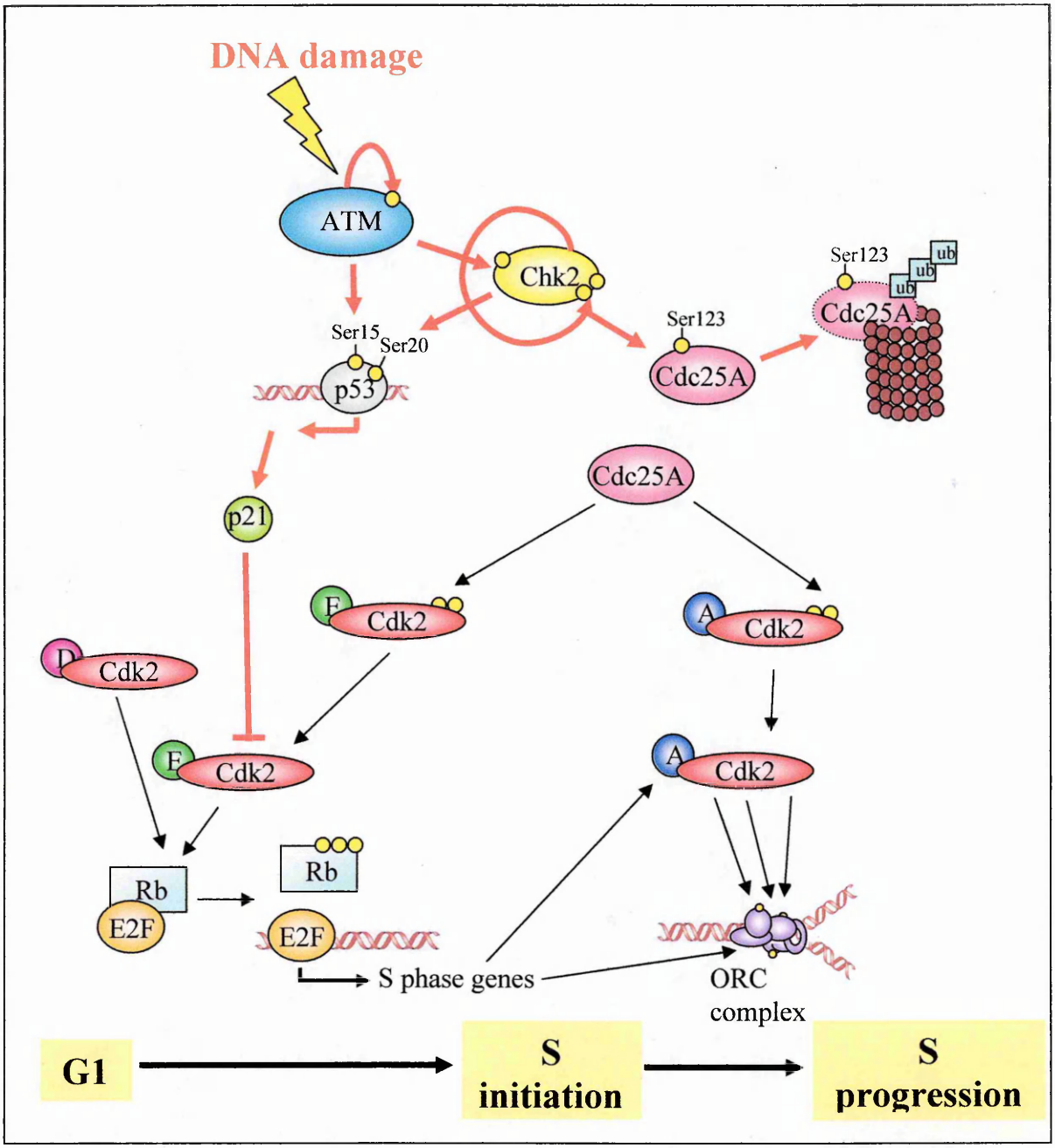

FIGURE 9: ATM and Chk2 dependent G1-S and intra S phase checkpoints. In presence of a DNA damage ATM and Chk2 are sequentially activated and could phosphorylate downstream substrates (red arrows). P53 is phosphorylated by ATM in Ser15 and by Chk2 in Ser20, both events leading to p53 accumulation and activation. $P21^{waf1}$ is transcriptional induced by p53 and is able to block Cdk2-CyclinE complex leading to an arrest of G1-S progression. Chk2 also phosphorylates Cdc25A phosphatase in S123 prompting Cdc25A to degradation. Cdc25A absence leads to a cell cycle arrest because this phosphatase in a normal cell cycle is essential for the dephosphorylation and activation of Cdk2-cyclin E complex in G1 cells, or Cdk2-cyclin A complex in early S cells.

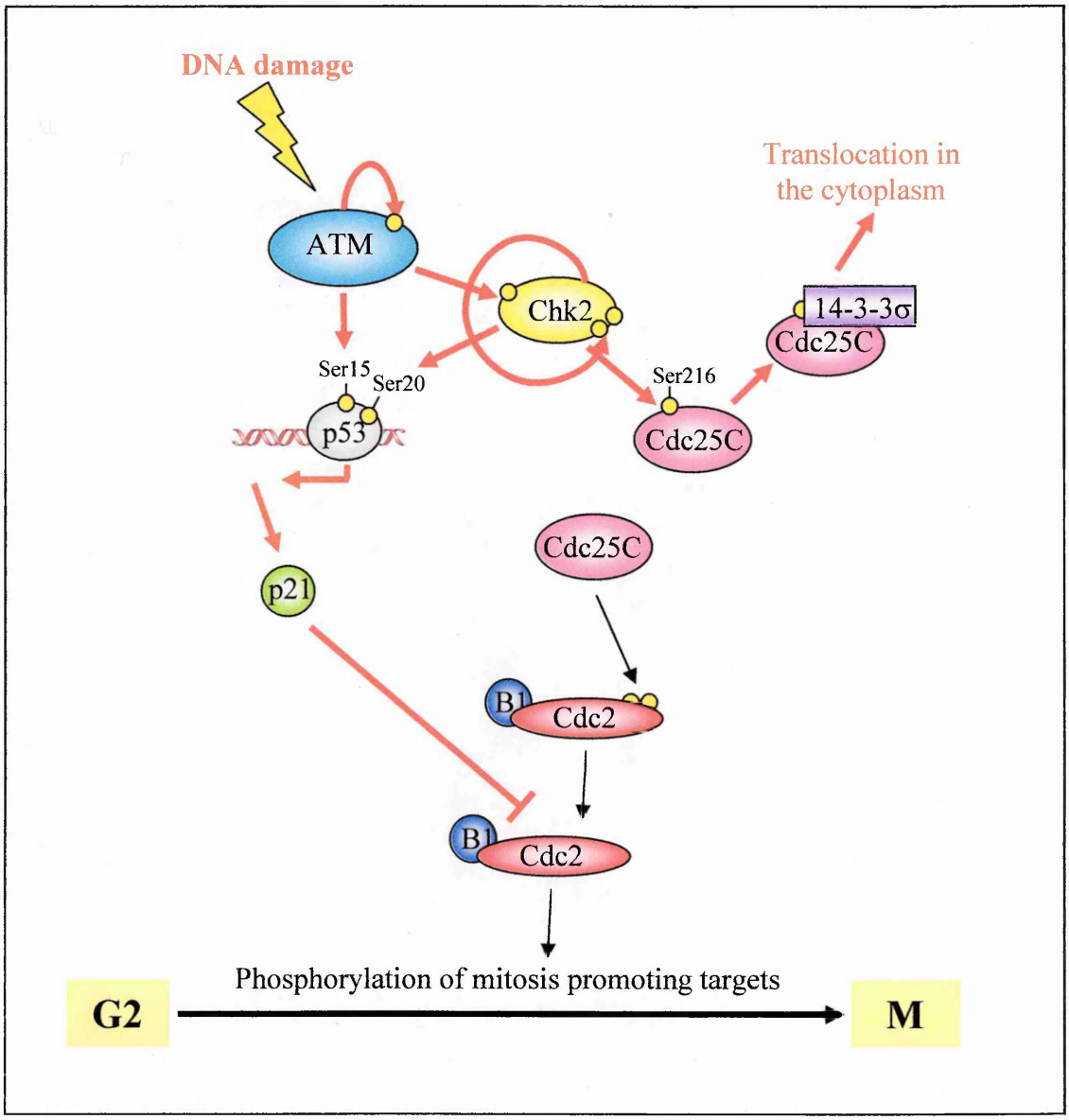

FIGURE 10: ATM and Chk2 dependent G2-M checkpoint. In presence of a DNA damage Chk2 is activated by ATM and, in coordination with ATM itself, phosphorylates p53 leading to accumulation and activation of this target. P53 transcriptionally induces many substrates, among them $p21^{waf1}$ that blocking Cdc2-cyclin B complex arrests G2-M progression. Chk2 reinforces G2-M checkpoint modifying Cdc25C and excluding this phosphatase from the nucleus, leading to accumulation of phosphorylated and inactive Cdc2-cyclin B complex.

AIM OF THE WORK

The main aim of this thesis was to elucidate of the mechanisms of Chk2 protein kinase activation in response to DNA damage, starting from earlier evidences showing that this kinase is activated by phosphorylation in an ATM-dependent manner in cells treated with ionizing radiation (IR).

In the first part of my thesis I investigated the role of Nbs1, a protein deficient in Nijmegen Breakage Syndrome (NBS), which shares with Ataxia Telangiectasia (AT) common clinical features including radiosensitivity. For this purpose I compared the phosphorylation and catalytic activity of Chk2 in cells derived from patients with AT and NBS with normal cell lines. Furthermore, using specific Nbs1 mutants I studied the role of the Nbs1/Mre11/Rad50 complex in this response.

In the second part of my thesis, because of the pivotal role of ATM and NBS1 in the cellular response to radiation, I evaluated the kinetics of Chk2 activation in normal, AT and NBS cell lines treated with IR, UV light and hydroxyurea.

DNA single and double strand breaks are the main lesions produced by IR as well as by oxidative stress. Having found that Chk2 is preferentially activated by IR, I analyzed the dependence of this event on ATM and Nbs1 proteins, and investigated Chk2 activation in relation to the amount of single and double strand breaks induced by IR and hydrogen peroxide. The yield of DNA breaks for each treatment dose was estimated by the neutral/alkaline elution and by γ-H2AX immunostaining. The activation of ATM and Chk2 kinases were evaluated using different *in vitro* and *in vivo* approaches to overcome limits in sensitivity by each assay. In this way I investigated the presence of lesion specificity for the ATM and Chk2 response, the importance of the source of damage when the lesions produced were comparable, and the possible presence of a threshold amount of DNA lesions necessary for a complete and sustained activation of each kinase.

MATERIALS AND METHODS

1. CELL LINES AND CULTURE PROCEDURES

1.1 Cell lines

Lymphoblastoid cell lines (LCLs) were derived from human lymphoblasts immortalized by Epstein-Barr virus (EBV) infection. LCL-N, LCL-N1 and LCL-N2 were obtained from normal individuals, AT52RM from an Ataxia Telangiectasia (AT) patient, 524RM and 227RM from two AT-heterozygotes, 1548 from an Italian Nijmegen Breakage Syndrome (NBS) patient. GM07078 (NBS patient), GM08036 and GM08037 (NBS heterozygous parents) were obtained from the Coriell Cell Repository (Camden, N.J.).

FB-N and 18ATRM are established fibroblasts from a normal individual and an AT patient, respectively. GM07166 are established fibroblasts derived from the same NBS patient as GM07078) and were obtained from the Coriell Cell Repository (Camden, N.J.).

ILB1 are SV40-immortalized NBS fibroblast, GM07166/NBS1 are fibroblasts stably transfected with the full length NBS1 cDNA, GM07166/s590 are stably transfected cells with the NBS1 truncated cDNA at codon 590, and ILB1/S343A are stably transfected with NBS1 with a serine to alanine substitution at aa343. Immortalized fibroblasts were obtained from Dr. Komatsu and Dr. Khanna laboratory.

MCF-7 cells are derived from a human breast adenocarcinoma tissue, while HCT116 and HCT116 $Chk2^{-/-}$ are wild type and Chk2 null colon cancer cells and were obtained from Drs. B. Vogelstein and F. Bunz (Johns Hopkins Cancer Center, Baltimore, MD).

1.2 Maintenance of cell lines

Cells were maintained at 37° C and 5% CO_2 in a Heraeus humidified incubator and all procedures were carried out in a Laminar flow hood.

The LCL cell lines were cultured in suspension in flasks using with RPMI 1640 medium supplemented with 15% heat inactivated fetal calf serum (FCS), penicillin (100U/ml) and streptomycin (100μg/ml). The medium was changed twice a week, unless otherwise indicated in specific experiments.

Fibroblasts and MCF-7 were maintained in adhesion in Petri dishes with DMEM medium supplemented with the same serum and antibiotics concentrations as for lymphoblastoid cells. HCT116 wild type and knock out cells were cultured in McCoy's 5A medium with 10% fetal bovine serum and antibiotics as above. Transfected GM07166 were maintained in medium supplemented with 100 μg/ml hygromicin, while transfected ILB1 were cultured with 500 μg/ml geneticin.

Cells were routinely passaged when they became confluent (every 2-3 days). The cell monolayer was washed with phosphate buffered saline (PBS) to remove serum (which inactivates trypsin) and then 2-6ml of 0.1% Difco Trypsin/EDTA (AnalaR BDH) was added to each flask and incubated for 5 minutes at 37° C. When cells had detached from the flask, trypsin was inactivated by adding 5ml of standard growth medium, and the cell suspension was pelleted by centrifugation at 1500 rpm for 8 minutes. The cells were then resuspended in standard growth medium and re-plated at an appropriate split ratio (1:3-1:5) for the cell line.

All cell lines used were routinely tested and found to be free of mycoplasma contamination.

1.3 Storage and retrieval of cells in liquid nitrogen

Frozen cell stocks in liquid nitrogen were routinely prepared. Lymphoblastoid cells were directly collected while fibroblast were trypsinized and in both cases resuspended in standard growth medium containing 10% dimethylsulphoxide (DMSO). 1ml aliquots were

frozen in cryotubes (L.T.I.) in a -80°C freezer for at least 24hrs, and then stored submerged in liquid nitrogen.

Cells were recovered from liquid nitrogen by thawing rapidly in a 37°C water bath and washing with 10ml of standard growth medium to remove the DMSO. Cells were centrifuged at 1500rpm for 8 minutes, resuspended in fresh medium and plated.

2. IRRADIATION AND TREATMENTS

2.1 Irradiation of cell cultures

Cells were γ-irradiated using a ^{137}Cs source emitting a dose rate of 8 Gy/min. Routinely cells were irradiate at room temperature, for the evaluation of the DNA strand breaks produced by ionizing radiation exposure the cells were irradiate on ice.

2.2 Hydrogen peroxide treatments

The cells were deprived of the culture medium (conditionated medium) by centrifugation and the conditionated medium was stored at 37°C. Cells were exposed for 1hr to the indicated hydrogen peroxide concentration (Sigma, Italy) in PBS solution at 37°C. After this period each sample was washed twice with PBS and resuspended in its own conditionated medium.

2.2 4-nitroquinoline 1-oxide (4-NQO) and Hydroxyurea (HU) treatments

4-NQO UV mimetic drug (Sigma, Italy) in a stock solution of 2mM in ethanol was added to the cells washed from medium and resuspended in PBS, for 1hr at the indicated concentration. After this period cells were washed twice with PBS and resuspended in fresh 37°C culture medium, 1hr later total lysates were prepared or cells were cytospun on glass slide for immunofluorescence staining.

HU (Sigma) was dissolved in PBS at a concentration of 500mM and added to cells at the indicated concentration for 24 or 48hrs. After this period cells were washed twice with PBS and total lysates were obtained. A part of the sample was prepared for cytometric analysis as described above.

3. PROTEIN ANALYSIS BY SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine changes in the expression or migration of proteins. This technique is a method by which proteins can be separated based on their molecular weight by the relative distance they migrate through a polyacrylamide gel matrix.

3.1 Sample preparation

Cells were collected and washed twice with PBS buffer supplemented with 0.1 mM Na_3VO_4 (Sigma), a phosphatase inhibitor. Cells were pelleted at 5000 rpm 10 minutes and lysed in 10µl/1x10^6 cells of Laemmli buffer (0.125M Tris-HCl, pH 6.8, 5%SDS). Lysates were boiled at 95°C for 5min, placed on ice and when at room temperature an equal volume of Laemmli buffer containing as inhibitors 1mM PMSF, 1µg/ml pepstatin, 100KIU/ml aprotinin, 10µg/ml leupeptin (all from Calbiochem)1mM EDTA, 10mM NaF and 1mM Na_3VO_4 was added. The suspensions were sonicated for 30sec. to dissolve DNA and quantified by the micro-BCA method (Pierce, Rockford, IL). Samples were stored at -80°C.

3.2 Preparation of acrylamide gels

The gel apparatus used in these experiments was a Hoefer Mighty Small II. A 7.5cm running gel (see Table) with a 2cm stack was prepared, using a 0.75mm spacers and well

former. To prepare the gel, a mix of separating buffer 1X (see table), APS, Temed and polyacrylamide/bis-acrylamide 29:1 at the necessary concentration was prepared and pour between two glass slides separated by two 0,75 mm flat spacer so that the level is 2 cm below the comb. A 10% running gel was used for optimum resolution of the p53 protein, a 8% was used for Chk2 and Nbs1, a 5% for ATM and a 12.5% for $p21^{waf1}$. All proteins required a 4.5% stacking gel prepared with a stacking buffer (see Table) in addition to the running gel.

3.3 Samples loading

The prepared lysates were thawed and 40µg of total protein in 5-20µl of Laemmli buffer were used for each sample. 10X loading buffer (Tris HCl 0,25 M, SDS8%, glycerol 30% and bromophenol blue 0,2% pH 6,8) was added to a final 1X concentration. The samples were boiled at 95°C 5 minutes and microfuged briefly to pellet any debris before loading onto the gels. In addition, 7µl of prestained Rainbow molecular weight marker (14.3-220kDa; Amersham, UK) was also loaded in a lane. Gels were run for approximately 5 hours in running buffer (see Table) initially at 50 V and then at 70-80V, until the dye front reached the end of the gel. For Chk2 band shift analysis a 12cm long gel was used and the run was extended up to 8 hours to a better separation of the phosphorylated forms of the protein.

3.4 Western transfer and immunoblot

Proteins were transferred onto a polyvinylidine difluoride (PDVF) membrane (Immobilon; Millipore), a microporous membrane with high protein binding capacity.

After discarding the stacking gel, the running gel was soaked in ice-cold transfer buffer for 10 minutes to remove the SDS. The Immobilon membrane is extremely hydrophobic and was therefore pre-soaked in 100% methanol for 5 minutes, and then

rinsed thoroughly in distilled water to remove the methanol. Finally membranes were equilibrated in transfer buffer for 10 minutes.

The proteins were transferred from the gel to the Immobilon membrane by a Semidry Electroblotting (BioRad) at 90mA for 1hour. Equilibrated PVDF membrane was put over two pieces of 3MM paper wet with transfer buffer. The gel was put over the PVDF membrane and overload with other two pieces of wet 3MM paper. Usually transfer was performed at 100-120mA for 1hour.

The resulting membrane was first washed 2 times with 50mM Tris-HCl pH 7.5, then equilibrated in PBS+0.1% Tween20 and finally blocked for 20 minutes in milk blocking buffer (see Table) at RT. The primary antibody was added to the membrane in 10ml of milk blocking buffer and the incubation was performed over night at room temperature. After incubation unbound primary antibody was removed with three washes of 10 minute in PBS+0.1% Tween20. The only exception was done for the phosphospecific antibody T68P that was diluted in PBS+3% BSA in presence of 0.1% Tween20, this to avoid the interference of serum phosphoprotein in epitope recognition.

Incubation with the secondary antibody was performed in a similar way as described for the primary antibody. In all cases, anti-mouse or anti-rabbit IgG horseradish peroxidase conjugate (Amersham), were used at a concentration of 1:2000. The membrane was incubated with the secondary antibody for 1 hour at room temperature and then washed three times for 10 minutes PBS+0.1% Tween20.

When a reprobe was necessary a treatment with 0.1% sodium azide, to eliminate the residual peroxidase signal and extensive washings in PBS+0.1%Tween20, were utilized.

3.5 The ECL detection system

Proteins were detected using the Enhanced Chemiluminescence (ECL) detection system. This is a rapid, highly sensitive, non radioactive method for the detection of immobilized proteins. In the ECL reaction, the substrate luminol is oxidised by a

horseradish peroxidase catalysed reaction resulting in blue light emission. This can be detected using blue light sensitive film. The immunoreactive bands were visualized by ECL Super Signal (Pierce, Rockford, IL.) on autoradiographic films and quantified by optical densitometry using a DuoScan system (Agfa, Mortsel, Belgium) and ImageQuant software (Molecular Dynamics, Sunnyvale, CA.).

3.6 Antibody Concentrations

The membranes were incubated with specific antibodies for Chk2 (a rabbit antibody kindly provided by Dr. Nakanishi and used 1:200 and a mouse antibody, clone 44D4, produced with standard techniques), Chk2 phospho-Threonine 68 (1:200 kindly provided by Dr. Chen), Serine 19 (Cell Signaling, 1:800), Serine 33/35 (Cell Signalling, 1:1000), Nbs1 (NB 100-143 polyclonal Novus Biologicals, Littleton, CO., 1:1000), p53 (DO-7, DAKO 1:1000), p53-P-Ser15 (clone 16G8, Signal Transduction, 1:200), $p21^{wafl}$ (Pharmingen BD, San Jose, CA., 1:100), actin (Sigma, Italy, 1:10000) and ATM (clone 4D2 produced with standard techniques, 1:800).

3.7 SDS-PAGE western blot solutions

10x loading buffer

0.25M Tris-HCL pH 6.8

8% (w/v) SDS (AnalaR)

36% (w/v) glycerol

20% (w/v) β-mercaptoethanol (Sigma)

0.2% bromophenol blue

10x Running buffer

1.9M glycine (AnalaR BDH)

250mM Tris (AnalaR BDH)

1% (w/v) SDS

4x separating buffer

1.5M Tris pH8.8

SDS 0.4%

4x stacking buffer

0.5M Tris pH6.8

SDS 0.4%

Transfer buffer

1.9M glycine (AnalaR BDH)

250mM Tris (AnalaR BDH)

20% (w/v) methanol (AnalaR BDH)

Blocking buffer

10mM Tris-HCL pH 7.4

150mM NaCl

Tween 0.1% (w/v) (Sigma)

Milk 5% (w/v)

4. GST RECOMBINANT PROTEINS PRODUCTION

The Cdc25C cDNA fragment encoding aa 200-256, kindly provided by Dr. Nakanishi, was cloned in the pGEX-4T-1 vector, in frame with the GST tag and finally used to transform the *E. coli* strain BL21.

To induce protein production, an over night culture was diluted 1:10 in fresh medium supplemented with ampicillin, and grown to an $O.D._{600}$= 0.5. Next IPTG was added to a final 0.1mM concentration and the growth continued for other three hours. Cells were then pelleted, washed with STE buffer (10mM Tris-HCl pH8, 150mM NaCl, 1mM EDTA) and resuspended in 4 ml of STE for each liter of culture. Lysozime was added to *E. coli* colture to a final concentration of 200μg/ml and left to digest on ice for 15 minutes, next 5 mM DTT, PMSF and 1.5% sarkosyl were added and samples frozen at –80°C. Bacterial cells were still lysed by repeated freeze-thawing cycles and sonication and the suspensions were clarified by centrifugation at 13000 rpm for 15 minutes. Surnatant was then applied to Glutathione-Sepharose 4B beads (Amersham-Pharmacia, 500 μl of slurry for each liter of bacterial culture) previously washed three times with PBS and once with PBS 1% Triton and incubated for 1 hour at 4°C with shaking. Beads were then washed 8 times with PBS and proteins were eluted over night at 4°C with 1 ml of 20mM Glutathione in PBS 0.1% Triton pH8. Purificated proteins were then quantified by SDS-PAGE and blue-coomassie staining (GEL-CODE, PIERCE).

5. IMMUNOPRECIPITATIONS AND *IN VITRO* KINASE ACTIVITY

5.1 Immunoprecipitations and phosphoresidues detection

For analysis of Chk2 phosphorylations in threonine 68 and threonine 387 residues two phosphospecific antibodies were used to evaluate the fraction of specifically modified in total Chk2 immunoprecipitated samples.

Lymphoblastoid cells were lysed on ice for 30 minutes in a lysis buffer containing 150mM NaCl, 1mM EDTA, 20mM Tris (pH 8), 0.5% Nonidet P-40 plus protease and phosphatase inhibitors (1mM PMSF, 1μg/ml pepstatin, 2μg/ml leupeptin, 2μg/ml aprotinin, 25mM NaF, 1mM Na_3VO_4). Lysates, clarified by centrifugation (10000rpm 10 minutes), were quantified with Bradford assay (Biorad). The same amount of proteins for each sample (1mg of total lysate) was precleared with 10μl of Sepharose-Protein G (Sigma) for 1hr and immunoprecipitated with 5μg of mouse 44D4/21 anti-Chk2 antibody and 10μl of Sepharose-Protein G at 4°C for 2hrs. After extensive washes the immunoprepitated molecules were solubilized in 20-30 μl in the same sample buffer used for SDS-PAGE, boiled for 10 minutes and centrifuged. Supernatants were analyzed by SDS-PAGE, and the phosphorylations on specific residues were revealed by pT68 and pT387 phosphospecific antibodies (Cell Signaling Tech., Beverley, MA) used, respectively 1:800 and 1:300.

For the analysis of serine 1981 phosphorylation event on ATM protein, cells were lysed in a buffer containing 50mM Tris-HCl, pH 7.4, 1% Tween-20, 0.2% NP-40, 150mM NaCl, 1mM EDTA plus protease and phosphatase inhibitors. Immunoprecipitations were performed with the same protocol as for Chk2 using the monoclonal anti-ATM antibody clone 4D2. Immunoprecipitated protein were run on a gel and immunoblotted with a phosphospecific antibody against S1981 (Rockland) at 1:200 dilution.

5.2 ATM and Chk2 kinase assay

To evaluate Chk2 kinase activity, cells were washed three times with PBS supplemented with 1mM Na_3VO_4 and lysed on ice for 30 minutes in a buffer containing 50mM Tris-HCl, pH 7.4, 0.2% Triton X-100, 0.3% NP-40, 150mM NaCl, 1mM EDTA plus protease and phosphatase inhibitors (1mM PMSF, 1μg/ml pepstatin, 2μg/ml leupeptin, 2μg/ml aprotinin, 25mM NaF, 1mM Na_3VO_4). The clarified lysates were quantified with Bradford assay (Biorad). The same amount of proteins (1-2mg of total lysate) for each sample was precleared with 10μl of Sepharose-Protein G (Sigma) for 1hr and immunoprecipitated with 5μg of mouse 44D4/21 anti-Chk2 antibody and 10μl of Sepharose-Protein G at 4°C for 2hrs. After extensive washes Chk2 kinase reactions were performed on immunoprecipitates at 30°C for 30min in 20μl reaction mixtures containing 50mM HEPES, pH 8.0, 10mM $MgCl_2$, 2.5mM EDTA, 1mM dithiothreitol, 10μM β-glycerophosphate, 1mM NaF, 0.1mM Na_3VO_4, 0.1mM PMSF, 10μM ATP, 20μCi [γ-^{32}P]ATP and 1 μg of GST-Cdc25C fragment (aa 200-256) as a substrate. To stop the reaction samples were supplemented with 4μl of loading buffer and boiled 5' at 96°C. The reaction products were separated on SDS-PAGE, and after autoradiography to evaluate [γ-^{32}P] incorporation, were Western blotted for Chk2 to verify the amount of immunoprecipitated protein per sample. To evaluate Chk2 autophosphorylation the GST-Cdc25C substrate was omitted in the kinase reaction.

ATM was immunoprecipitated from cells lysed in 50mM Tris-HCl, pH 7.4, 1% Tween-20, 0.2% NP-40, 150mM NaCl, 1mM EDTA plus protease and phosphatase inhibitors, using the monoclonal anti-ATM antibody clone 4D2, and kinase reactions performed in a buffer containing 20mM HEPES, pH 8.0, 50mM NaCl, 10mM $MgCl_2$, 10mM $MnCl_2$, 1mM dithiothreitol, 20μM ATP, 10μCi [γ-^{32}P]ATP and 1μg PHAS1 substrate.

6. NEUTRAL AND ALKALINE ELUTION ASSAY FOR DNA SSB AND DSB

Cells were labelled with 0.08 μCi/ml 2-^{14}C thymidine for 24hrs at 37°C. After removal of the radiolabelled nucleoside precursor, cells were incubated for a further 24hrs prior to treatment with hydrogen peroxide or irradiation (the latter performed on ice). The neutral and alkaline elution procedures have been described elsewhere (Kohn *et al.*, 1981). Briefly, after genotoxic treatments, the cells were layered on 2μm pore polycarbonate filters, lysed with a solution containing 2% SDS, 0.1M glycine, 25mM disodium EDTA (pH 10 for SSB, pH 9.6 for DSB) and 0.5mg/ml proteinase K, and the DNA eluted with a solution containing 0.1% SDS, 20mM EDTA (acid form) adjusted to pH 12.15 (for SSBs) or 9.6 (for DSBs) with tetrapropylammonium hydroxide. During the 15hrs of the elution procedure, fractions were collected and radioactivity measured by liquid scintillation. To allow a comparison between samples, the amount of DNA damage was converted into a fragmentation index (FI) based on the formula ($1-r/r_0$, where r and r_0 are the fractions of [^{14}C] DNA remaining on filters of treated and untreated cells, respectively), calculated after 12hrs of elution.

7. IMMUNOFLUORESCENCE TECHNIQUES

7.1 Detection of γ-H2AX nuclear foci

Cells were cytocentrifuged (500 r.p.m., 7') onto glass slides using a cytospin instrument (Shandon), air dried over night. Fixation was performed for 20min at –20°C in ice cold methanol, and thereafter in acetone (2' at –20°C). After washing with PBS and blocking for 1hr with 3% BSA, the slides were incubated for 2hrs with 1:400 dilution of an anti phospho-H2AX (Ser-139) antibody (clone JBW301, Upstate Biotechnology) and thereafter with an $F(ab)_2$ fragment of a FITC-conjugated secondary antibody for 1hr. After

3 washes in PBS, slides were counterstained with DAPI and mounted with an antifade solution (PBS + 75% glycerol + 0.2M DABCO). Images were collected using a Zeiss Axioskop fluorescence microscope and digital imaging. Nuclear foci were enumerated by two independent operators, on experiments performed three independent times and on duplicate slides.

7.2 Mre11 and total Nbs1 immunostaining

The indirect immunofluorescence staining of cytospin preparations for Nbs1 and Mre11 was performed as reported (Carney *et al.*, 1998) using 1:50 to 1:100 dilutions of rabbit antibodies to NBS1 and MRE11 (both from Novus Biologicals). Nuclei were counterstained with DAPI.

7.3 Chk2 immunostaining

Immunofluorescence painting for Chk2 was performed on cytospins fixed for 10 min in 2% paraformaldehyde, washed in TBS, permeabilized with 0.1% Triton X100 and incubated with 1:50 dilution of a rabbit anti-Chk2 antibody (Santa Cruz Biotech.). A normal rabbit serum was used as a negative control. Binding of primary antibodies was revealed with a FITC-labelled Fab_2 goat anti rabbit antibody (1:50 dilution). Coverslips were mounted with the anti-fade solution supplemented with the DNA counterstain DAPI. The immunofluorescence specificity of Chk2 antibody was verified by competition with a recombinant full length Chk2 protein.

7.4 Detection of phospho-Thr68 Chk2 and phosphor-Ser343 nuclear foci

The immunofluorescence painting of the Chk2-Thr68 phosphorylated form was performed as described in Ward *et al.*, 2001 and using a phosphospecific antibody kindly provided by Dr. Chen or using an antibody purchased from Cell Signaling Technology (Beverley, MA).

The Nbs1-S343 phosphorylated foci were detected using the same technique described in Gatei *et al.*, 2000 and a phosphospecific antibody kindly provided by Dr. Khanna.

8. RADIORESISTANT DNA SYNTHESIS ASSAY

The rate of DNA synthesis was measured by the ^{14}C-/^{3}H-thymidine double labeling method. Cells were prelabeled with 10 nCi/ml ^{14}C-thymidine (Amersham Biosciences, UK) for 24 hours, then washed and incubated for 6 hours in non radioactive medium. Thereafter, cells were exposed to varying doses of γ-rays and immediately seeded in a 96-well plate in quadruplicates for a further incubation of 1 hour. Finally cells were pulse labeled with ^{3}H-thymidine at 2 μCi/ml for 15 minutes. Cells were washed with PBS, trypsinized, trapped onto glass microfiber filters (Whatman GF/C), rinsed twice with an ice-cold 10% trichloroacetic acid, then with ice-cold ethanol and acetone, and air-dried. The radioactivity of each sample was quantified by a liquid scintillation counter (Packard TriCarb 2200CA) and the $^{3}H/^{14}C$ ratio (corrected for those c.p.m. that were the results of channel crossover), was taken as the rate of DNA synthesis. The ratio of incorporated ^{3}H to ^{14}C was used for quantification to standardize the variation in DNA recovery. Quadruplicates were used to minimize the standard error of measurements.

9. FLOW CYTOMETRY PROCEDURES

Flow cytometry was used to determine the percentage of cells in the various phases of the cell cycle according to the DNA content.

9.1 Propidium iodide (PI) staining for assessment of DNA content.

Single colour flow cytometric analysis of DNA content by PI staining was carried out essentially as described by O'Connor *et al.* (1993). Cells were trypsinized, washed in ice-cold PBS, and aliquoted at 1 x 10^6 cells per ml in PBS into FACS tubes (Falcon). The cells were pelleted by centrifugation at 3000rpm for 3 minutes, fixed in 1ml 70% ethanol and stored at -20° C for at least 24 hours. Cell pellets were vortexed before incubating in 1ml PBS with 150 μg/ml RNaseA (Sigma) and 20 μg/ml propidium iodide (Sigma) at 37° C for 30 minutes. Stained cells were kept at 4° C in the dark overnight before analysis on a FACScan (Becton Dickinson). For all procedures, cells were excited with a single 488-nm argon laser and PI fluorescence was detected through a 585± 22nm filter. Red fluorescence data (FL3-Area and FL3-Width) were collected in list mode format to 10,000 total events using the LYSYS II program. Cell cycle analysis was carried out using the CellFIT program on FL3-A histograms (gated from cytograms of FL3-Area and FL3-Width, to exclude clumped cells), and the percentage of cells in G0/G1, S and G2/M were calculated using the rectangular fit (RFIT) model.

RESULTS

1. NBS1 DEPEDENCE OF CHK2 ACTIVATION AFTER DNA DAMAGE

1.1 Chk2 phosphorylation in normal cell lines

Human Chk2 protein, following modifications of the phosphorylation status, changes its electrophoretic mobility in western blot analysis (Brown *et al.*, 1999; Matsuoka *et al.*, 1998). To test the kinetics of this event in a normal background I performed time-course western analysis on extracts from exponentially growing human cells before and after 4Gy of ionizing radiation. In four different cell lines, two normal lymphoblastoid (LCL-N1 and LCL-N2), a normal fibroblast (FB-N), and a breast cancer (MCF-7) with apparently normal ATM-dependent radiation response, the anti-Chk2 immunoreactive band present before irradiation, underwent a stepwise delay in electrophoretic mobility between 30min and 3hrs after treatment (Figure 11A).

A detailed time-course analysis on LCL-N cells showed that Chk2 mobility maximally decreased between 1 and 3hrs post IR and remained apparently unchanged for up to 10hrs, thereafter the slow migrating Chk2 molecules decrease substantially to become undetectable at 72hrs (Figure 11B). As Chk2 electrophoretic retardation is due to phosphorylation since it can be eliminated by treatment with phosphatases (Matsuoka *et al.*, 1998, and data not shown), these findings indicate that Chk2 is phosphorylated in more than one site, that the full phosphorylation takes place within 1hr of irradiation and appears to involve most, if not all, Chk2 molecules. It should be underlined that the modifications of Chk2 phosphorylation detected in the early hours post irradiation were not due to cell cycle phase redistribution, as demonstrated through a flow cytofluorimetric analysis of single cells DNA content (data not shown).

1.2 Role of ATM in Chk2 phosphorylation after ionizing radiation treatments: analysis of AT patients and mutated ATM carriers

In budding and fission yeast the activation of Rad53 and Cds1 is dependent on the Mec1 and Rad3, respectively (Brown *et al.*, 1999; Chaturvedi *et al.*, 1999; Matsuoka *et al.*, 1998). Considering that ATM and Chk2 are respectively the human homologs of Mec1/Rad3 and Rad53/Cds1, I analysed the regulation of Chk2 in the lymphoblastoid AT-derived cell line AT52RM negative for ATM protein expression (Delia *et al.*, 2000). In AT52RM, and in contrast to LCL-N cells, no Chk2 phosphorylation was seen at any time-points post IR (Figure 12), thus confirming previous results and validating the experimental conditions used here to detect ATM-dependent Chk2 phosphorylation changes.

In consideration of the high number of ATM mutation carriers (more than 1% of the total population) and the debate about the cellular defects and their predisposition to cancer, I assessed whether a reduced expression of ATM is sufficient to cause perturbation in Chk2 phosphorylation employing the AT-heterozygous cell line 227RM (ATM wt/mut), which exhibits 30% of the normal levels of ATM protein (Delia *et al.*, 2000). The IR-induced phosphorylation of Chk2 in this cell line, analyzed as Chk2 mobility shift retardation, was comparable with normal cells (Figure 12); this observation was further confirmed in two others ATM heterozygous with less than 50% protein level (data not shown). These findings demonstrate that Chk2 is rapidly phosphorylated after 4Gy DNA damage in an ATM-dependent manner and even in ATM haploinsufficient background.

1.3 NBS1-dependent Chk2 phosphorylation after DNA damage

1.3.1 Chk2 phosphorylation in NBS patients and mutated NBS1 carriers

The overlapping clinical and cellular abnormalities between AT and NBS prompted us to determine the impact of NBS1 gene alterations on Chk2 regulation.

The NBS-derived lymphoblastoid cell lines 1548 and GM07078A, homozygous for two different truncating mutations of the NBS1 gene (Cerosaletti *et al.*, 1998; Tupler *et al.*, 1997; Varon *et al.*, 1998) were, unlike normal and AT cells, both negative for Nbs1 full length protein (Figure 13A). 1548 cell line is derived from an Italian patient and shows a rare mutation (835del4), GM07078A is obtained from a Slavic patient and carries the most frequent NBS1 mutation described (657del5) associated with a founder effect.

In these NBS cells, and in marked contrast to normal cells, no delay in the electrophoretic mobility of Chk2 (Figures 13B and 13C) was seen at any time points after IR, with the exception of a transient but modest shift at 3hrs which suggested that a small fraction of Chk2 molecules may have undergone a partial phosphorylation at this time point. These findings suggest that the phosphorylation of Chk2 is markedly impaired by Nbs1 deficiency and emphasize the role of Nbs1 for the rapid and sustained phosphorylation of Chk2.

As for ATM, two NBS carriers expressing >45% of the normal Nbs1 levels (data not shown) had no defects in IR-induced Chk2 phosphorylation (Figure 13D), indicating that, like ATM, NBS1 haploinsufficiency plays no effect on Chk2 responses.

ATM phosphorylates preferentially serines and threonines followed by a glutamine. Clusters of SQ and TQ residues are present in many ATM targets (Abraham *et al.*, 2001), including Chk2 which contains six such residues. The phosphorylation of one of these residues, T68, is an essential event for Chk2 activation (Ahn *et al.*, 2000), although phosphorylation of other residues, eg: S33, S35 and S19, provides a strong and sustained Chk2 activity (Buscemi *et al.*, 2006). With the use of commercially available

phosphospecific antibodies I confirmed the data obtained in western blot analyses by mobility shift assessment. Indeed, NBS cells revealed no detectable phosphorylation of S19 and S33/S35 in response to 4Gy IR, whereas phosphorylation of T68 was largely reduced (< 50%) compared to normal cells (Figure 14), thus confirming that Nbs1 deficiency impairs Chk2 phosphorylation.

1.3.2 Chk2 phosphorylation in NBS cells complemented with normal or mutated forms of Nbs1

To verify that the defective Chk2 regulation in NBS cells was entirely due to Nbs1 protein deficiency, and not to other additional defects present in the genetic background of these cell lines, I analysed Chk2 in NBS cells complemented with wild type NBS1.

For this approach I used fibroblasts (GM07166) derived from the same patient of GM07078, and SV40 transformed immortal NBS fibroblasts (ILB1), established from primary cells derived from a Polish patient, carrying the common Slavic founder mutation 657del5. Both cell lines were stably transfected with an empty eukaryotic expression vector or with the same vector containing wt or mutated forms of the NBS1 gene.

To further define the role of NBS1 in Chk2 response, we analysed also NBS cells transfected with an NBS1 cDNA encoding a protein truncated at aa590 (GM07166/s590) that deletes the carboxy-terminus region containing the Mre11 binding domain (Tauchi *et al.*, 2001). We also tested an NBS1 mutant containing a serine-to-alanine substitution at position 343 (ILB1/S343A), a site directly phosphorylated by ATM immediately after DNA damage. Mutations in serine 343 are known to only partially complement the S-phase checkpoint in NBS cells (Gatei *et al.*, 2000; Lim *et al.*, 2000). Cell lines containing these constructs were obtained from Dr. Tauchi and Dr. Khanna laboratories. The western blot analysis of lysates obtained from GM07166 and ILB1 cell lines stably transfected verified that the ectopic wild type and mutants proteins were correctly expressed and showed the expected molecular sizes (Figure 14).

The same mutants were also characterized for Nbs1, Mre11 and Chk2 proteins expression and localization by immunofluorescence of cytospin preparations to determine the relationship between NBS1 status, NBS1-complex formation and Chk2 localization. NBS1 protein, undetectable in NBS cells (Figure 16, left panel, E-F), demonstrated a nuclear fluorescence both in normal (A, B) and NBS cells ectopically expressing wild type (C-D) or mutant s590 (G-H) and S343A (I-L) Nbs1 proteins. Mre11 (Figure 15 middle panel) also shows a nuclear localization in normal (A-B) and NBS cells ectopically expressing wild type (C-D) or S343A NBS1 (I-L), whereas it is characterized by a diffuse, nuclear and cytoplasmic, fluorescence in NBS cells (E-F) and in NBS/s590 (G-H), the latter finding demonstrating that the C-terminus region of NBS1 is essential for the nuclear localization of the NBS1/Mre11/Rad50 complex (as confirmed in Tauchi *et al.*, 2001). Chk2 distribution in nuclear compartment that was detectable in normal cells was also retained in NBS cells, whether or not transfected with the various Nbs1 mutants (Figure 16 right panels). Likewise, the nuclear localization of Chk2 in these cells was unaffected by IR, even up to 8hrs after treatment (not shown). These unchanged nuclear distributions exclude a defect in Chk2 localization as the cause of its failed phosphorylation in NBS cells, and confirm the correct expression and localization of all the analyzed protein in NBS1 transfected cells.

Analysis of Chk2 protein in lysates obtained from NBS cells ectopically expressing wild type NBS1 (GM07166/NBS1 and ILB1/NBS1) showed, in contrast to that from parental or mock-transfected cells, a complete restoration of Chk2 phosphorylation in response to 4Gy IR (Figures 17A and B). Neither the C-terminus deletion mutant nor the S343 mutated NBS1 were able to efficiently complement the Chk2 phosphorylation defect (Figure 17C).

Since in NBS cells Chk2 phosphorylation is largely impaired and unsustained but can be complemented by a full length Nbs1 I can conclude that this event is NBS1-dependent. Together with immunofluorescence data, the results obtained with the NBS1 mutants

indicate that Chk2 phosphorylation is dependent on a nuclear localized, functional and ATM-phosphorylatable Nbs1-complex.

1.4 Nbs1-dependent Chk2 kinase activity.

To evaluate if Chk2 activity in addition to Chk2 phosphorylation was defective in cells from Nijmegen Breakage Syndrome patients, the catalytic activity of Chk2 was examined *in vitro*. I tested the incorporation of a radiolabelled phosphate in a fragment of Cdc25C containing the target residue Serine 216 in presence of Chk2 protein immunoprecipitated from normal and NBS cells extracts before, 30min and 3hrs after 4Gy of γ-radiation.

In normal lymphoblastoid cells a slight basal activity of Chk2 was detectable in untreated cells but increased up to ~5fold at 3hrs but not at 30min after IR treatment (Figure 15A). Conversely, in NBS cells no increase in Chk2 kinase activity was observed at any time points after IR (Figure 18A). Western blots of the same reactions verified the presence of similar amounts of immunoprecipitated Chk2 protein (Figure 18A). Importantly, ectopic expression in NBS cells of wild type Nbs1 restored the induction of Chk2 kinase activity in response to IR, to the same extent as in normal cells (Figure 18A). These results, in addition to those obtained by the mobility shift analysis, demonstrated that Nbs1 is necessary for the phosphorylative activation of Chk2 by IR. Moreover, the finding that Chk2 activity does not increase in normal cells at 30min and in NBS cells at 3hrs post IR (Figure 18B) in presence of a modest phosphorylation (Figure 11A), suggests that the activation of Chk2 depends on multiple phosphorylation steps.

2. TREATMENT- AND DOSE-SPECIFICITY OF CHK2 PHOSPHORYLATION

It was previously reported that whereas low doses of IR activate Chk2 in an ATM-dependent manner, high doses of IR activate Chk2 independently of ATM. Furthermore is

widely accepted that ATM activity is induced principally, if not exclusively, by damaging agents producing specific DNA lesions, that are double strand breaks. Therefore, I analyzed Chk2 phosphorylation in normal and NBS cells exposed to high doses of IR and DNA damaging agents that do not produce double strand breaks, in order to correlate Nbs1 activity on Chk2 specifically with ATM.

2.1 Chk2 phosphorylation after high dose of IR.

I analyzed Chk2 phosphorylation in NBS cells exposed to 50Gy of IR in the attempt to determine the Nbs1 dependence of this modification in response to extensive damage. In contrast to the findings observed after 4Gy of IR, the time-dependent phosphorylation of Chk2 in NBS and AT cells progressed as in normal lymphoblastoid cells LCL-N (Figure 19), hence indicating that Chk2 activation becomes NBS1-independent other than ATM-independent following large scale DNA damage.

2.2 Chk2 phosphorylation after different genotoxic treatments.

Exposure of cells to UVC induces as primary damage the formation and accumulation of dipyrimidine photoproduct that mainly activate the NER pathway to repair DNA (for a review see Araujo and Wood, 1999). To investigate the role of Chk2 in response to these lesions, fibroblasts, were exposed to 10 J/m^2 of UVC and examined for Chk2 modification. UV radiations are characterized by low energy and also a single cell can reduce the radiation received by the successive. This is the reason why fibroblasts, which grow in a monolayer, were chosen for this analysis. Compared to untreated controls, no Chk2 phosphorylation changes were detected in normal, AT and NBS cell lines at any time points after UV treatment (Figure 20A), despite the effectiveness of the dose used to elicit a DNA damage response as demonstrated by the ATM- and NBS-independent accumulation of p53 protein (Figure 20B).

These findings would thus seem to exclude the activation of Chk2 in response to pyrimidine dimers formation, although this conclusion is in apparent contrast with other reports (Matsuoka *et al.*, 1998; Tominaga *et al.*, 1999). This discrepancy could be explained by the fact that previous studies were based on the use of much higher doses of UV (50-700 J/m^2), that could induce DSBs and oxidative stress besides base modifications.

The genotoxic agent hydroxyurea (HU) rapidly inhibits ribonucleotide reductase and profoundly decreases purine pools, causing inhibition of replicative DNA synthesis and block of the replicative forks (Krakoff *et al.*, 1968; Snyder, 1984). To investigate the effect of HU, the cells were incubated in 1mM HU for 8, 16, 24 and 48hrs; and analysed for Chk2 status as well as for the cell cycle distribution using flow cytometry. No detectable changes in Chk2 electrophoretic mobility were found throughout the time course of HU treatment, except for a faint phosphorylation at 48hrs (Figure 21). The DNA flow cytometric analysis evidenced a cell cycle arrest in S-phase evident in normal NBS and AT cells at 24hrs (Figure 21), but not before this time point (data not shown), while at 48hrs a discrete amount of cells appeared dead (Figure 21). Thus, the finding that S-phase arrest induced by HU at 24hrs occurs without changes in Chk2 phosphorylation, would exclude the involvement of this kinase in the response to replication fork block.

3. DAMAGE SPECIFICITY OF CHK2 ACTIVATION

The differences detectable in Chk2 modifications after different sources of damage and following high doses of ionizing radiation prompted me to clarify the relation between ATM-Chk2 pathway activation and the amount and characteristics of the DNA lesions generated by genotoxic treatments. In particular, to correlate DNA backbone damage (single or double strand breaks) and ATM or Chk2 activation the first step was to evaluate in a sensitive approach the presence of DNA brakes. The role of other kind of lesions (i.e. pyrimidine dimers or arrested forks), already excluded by the absence of Chk2

modifications following low doses of UV or HU treatments, was also further explored by the analysis of UV mimetic treated cells.

3.1 SSBs and DSBs estimation in IR and H_2O_2 treated cells

Two different techniques were used to evaluate the induction of single and double strand breaks, alkaline elution and γ-H2AX immunopainting. Alkaline elution technique (AET) permits to discriminate SSBs and DSBs in the same sample evaluating the rate at which DNA strands are carried through a microporous filter by a fluid flow (Kohn *et al.*, 1981). DSBs were, in addition, monitored through the analysis of phosphorylated H2AX (γ-H2AX) nuclear foci in order to increase the sensitivity and the confidence with data obtained. Phosphorylated H2AX (γ-H2AX) is essential to the efficient recognition and (or) repair of DNA double strand breaks (DSBs), and many molecules, often thousands, of H2AX become rapidly phosphorylated at the site of each nascent DSB, spanning megabases around. A specific antibody to γ-H2AX reveals that this highly amplified process generates nuclear foci, and the number of these foci reflects that of DSBs (Rogakou *et al.*, 1999).

In LCL-N cells, SSBs determined by AET increased with the dose of IR, but even at the lowest dose tested (0.25Gy) their amount was rather marked, accounting for a fragmentation index (FI) of 40% (Figure 22). DSBs, on the other hand, became detectable by AET at doses of IR >2Gy (FI: 8% at 2Gy) (Figure 22), and their yield increased in a dose-dependent manner. As expected, massive amounts of SSBs and DSBs (FI: 100%) were seen after 50Gy. The yields of SSBs and DSBs in irradiated AT52 and NBS cells were close to those of LCL-N receiving similar treatment (data not shown). AET analysis was also applied to hydrogen peroxide (H_2O_2) treatments, in order to evaluate the ATM-Chk2 response in relation to another agent that induces DNA breaks. Particularly H_2O_2 is known to produce in a dose dependent way both single and double strand breaks, but with a higher rate of SSBs than IR (Birnboim and Sandhu, 1997; Guidarelli *et al.*, 1997).

Extensive SSBs were seen in LCL-N cells exposed to 20μM and 100μM H_2O_2 (FI: 50% and 100% respectively) (Figure 22), however DSBs were seen only in response to the latter concentration (FI: 40-60%) (Figure 22).

Although the AET is a useful technique enabling the quantification of both SSBs and DSBs from the same sample, its sensitivity limit doesn't allow the detection of DSBs induced by <2Gy IR, estimated to be <60 breaks per diploid cell (Rogakou *et al.,* 1999). As the average number of γ-H2AX foci per cell well compares with the number of induced DSBs (Rogakou *et al.*, 1999; Petersen *et al.*, 2001; Rothkamm and Löbrich, 2003; Furuta *et al.*, 2003; Jakob *et al.*, 2003), I quantified DSBs through the analysis of γ-H2AX foci. In LCL-N, the γ-H2AX foci per cells accounted for 2.8±0.9, but 5 min after irradiation with 0.25Gy, 0.5Gy and 1Gy their number rose to 7.8±0.4, 13.6±0.8 and 19.9±1, respectively (Figure 23). LCL-N cells treated with 20μM and 100μM H_2O_2 presented 6.9±0.5 and 21.4±1 γ-H2AX foci, respectively. By contrast, no γ-H2AX nuclear foci were seen in response to 4-NQO, a UV-mimetic compound generating base modifications and secondary SSBs, but not DSBs (Brosh *et al.*, 1999; Mirzayans *et al.,* 1999), thus supporting the specificity of this technique for DSBs. The damaging activity of 4-NQO was confirmed by an ATM-independent accumulation of p53 protein (data not shown). Concordant with other studies (Rogakou *et al.*, 1999), the foci per cell showed, in all samples, a Poisson distribution (data not shown). These data demonstrate that IR and H_2O_2 generate a dose-dependent number of DSBs, detectable even at the lowest doses tested (0.25Gy and 20μM H_2O_2), that are always accompanied by a preponderant amount of SSBs.

3.2 DSBs disappearance after DNA damage repair

DSBs repair in mammalian cells occurs as a two waves process, first a rapid response, within 1 hour, than a slow component, extending up to 24 hours, working on

more complex lesions (Woods, 1981; Price *et al.*, 2000). To further support my analysis on γ-H2AX, I estimated the number of foci 1hr after the exposure of cells to DNA damaging agents. When analyzed one hour after recovery from IR, the foci decreased to 4±1(0.25Gy), 10±1 (0.5Gy) and 12±2.1 (1Gy) (Figure 23), suggesting that at this time point not all lesions had been repaired (Nunez *et al.*, 1995). Also the number of γ-H2AX foci produced by H_2O_2 was markedly reduced 1hour after recovery from treatment (Figure 23), as a result of a repair activity.

To study the DSBs repair activity as function of the ATM-Chk2 pathway, I analyzed the γ-H2AX foci in response to 0.25-1Gy of IR in the ATM null cell line AT52 and in the Chk2 knock out cell line HCT116-Chk2-/-. In AT52RM (Figure 24A), the number of induced γ-H2AX was comparable with those seen in LCL-N cells in response to similar doses of IR. However, 1hr after recovery, the number of γ-H2AX foci in AT52 had not declined, in contrast to what observed in LCL-N cells (Figure 24A), indicating a defective early DNA repair in the absence of ATM. In the HCT116 parental cells, the γ-H2AX foci detected immediately after treatment (Figure 24B) were comparable with those in LCL-N cells and proportional to the dose of IR, and 1hrs after recovery the number was diminished by 40% (Figure 24B) a value similar to that already shown in LCL-N cells, consistent with a DNA repair activity. Likewise, in HCT116-Chk2-/-, the number of foci before and after recovery were essentially the same as in the parental HCT116 cells (Figure 24C), hence indicating that Chk2 deficiency does not alter the early DNA repair after low doses of IR in this cell line. Therefore in normal cells a first wave of DNA repair activity occurs within 1hr of the damage, resulting in almost 50% reduction of detectable DSBs-associated γ-H2AX foci, almost restoring the basal conditions in cells treated with 0.25Gy or 20μM H_2O_2. This early repair is grossly impaired in ATM but not in Chk2 null cell lines in response to the induction of a limited amount of double strand breaks. This final

observation is in accordance with the delayed activation of Chk2 detectable more than 1 hour after the DNA damage occurrence.

3.3 IR dose dependent activation of ATM

Exposure of cells to radiation triggers ATM kinase activity, enabling it to phosphorylate several substrates involved in multiple cell cycle checkpoints (Shiloh, 2003). The sensitivity, extent and speed of the ATM response were described as extremely high. Doses of irradiation that just cause two DSBs in a human cell activate the majority of ATM within minutes (Bakkenist and Kastan, 2003). I evaluated in LCL-N cells the *in vitro* and *in vivo* activity of ATM to determine its response to the initial amount of genomic damage. *In vitro* kinase assays were performed on cells harvested 30min and 3hrs after irradiation. At 30min, the basal activity of ATM increased in a IR-dose dependent manner starting from 0.25Gy and reaching a maximum of ~3fold increase in response to 2Gy or above (Figure 25A). However, at 3hrs no ATM activity was seen after 0.25 or 0.5Gy, whereas after 4Gy this activity was still elevated (Figure 25A), hence suggesting that doses of IR between 0.25-0.5Gy elicit a transient activation of ATM. To verify this possibility, I analysed the autophosphorylation of ATM Ser1981, an event reflecting the activation of ATM by DNA damage (Bakkenist and Kastan, 2003). Time-dependent analysis performed after 0.25 and 4Gy (Figure 25B) showed that the ATM pS1981 signal, whereas after 0.25Gy had increased 2.5 fold at 30min to drop to basal level at 3hrs, after 4Gy the signal increase at 30min was 5.0 fold and only slightly dropped at 3hrs (3.5 fold). These results, together with the *in vitro* kinase assays, indicate that while ATM is rapidly activated by very low levels of DNA breaks, only after the induction of a higher level of damage a significant residual ATM activity is detectable for an extended period after damage occurrence.

As an indication of the ATM activity *in vivo*, I assessed the phosphorylation of its target residues Nbs1-Ser343, Chk2-Thr68 and p53-Ser15 (Shiloh, 2003). Nbs1-Ser343 and

Chk2-Thr68 phosphoresidues, examined on cytospin preparations by immunofluorescence staining (respectively Figure 26A and 26B), showed in LCL-N a localization in nuclear foci, whose basal number increased starting from 0.25Gy. In AT cells, the basal number of phosphorylated Nbs1-S343 and Chk2-Thr68 foci did not increase even after 4Gy IR (Figure 26A and B), concordant with an ATM-dependence of these events. The specificity of Chk2-Thr68 phosphorylation was confirmed on immunoblots of Chk2 immunoprecipitated from untreated and treated cells (Figure 26C). As a third confirmation in LCL-N cells, the levels of p53-P-Ser15 at 30 min rose modestly after 0.25-1Gy, but substantially after 2-4Gy (Figure 26D). The defective phosphorylation of p53-Ser15 in irradiated AT cells verified the ATM-dependence of this event (Figure 26D and data not shown).

Collectively, these findings show that the ATM kinase is triggered by IR doses as low as 0.25Gy, yet this activity becomes sustained above 1Gy, and thus in response to a substantial number of DSBs.

3.4 Chk2 phosphorylation and activation after increasing doses of IR

Since the phosphorylation and activation of Chk2 are largely dependent on ATM (Brown *et al.*, 1999; Matsuoka *et al.*, 2000, Buscemi *et al.*, 2001), I examined these events as function of the amount of DNA damage. IR dose-dependence studies showed that the phosphorylation-related upward shift of Chk2, just detectable 3hr after 0.5Gy, was maximal after 2Gy (Figure 27A) and involved most of the Chk2 molecules. A similar Chk2 mobility shift was seen one hour after irradiation (data not shown). *In vitro* kinase assays on Chk2 immunoprecipitated from LCL-N cells to determine the correlation between mobility shift and enzymatic activity, showed an enhancement of the basal activity of Chk2 towards Cdc25C substrate only in response to 1Gy, but not to lower doses of IR (Figure 27B).

As a measure of Chk2 activity *in vivo,* the autophosphorylation of Chk2-Thr387 (Schwarz *et al.*, 2003) was evaluated on immunoblots of Chk2 immunoprecipitates from LCL-N, AT and NBS cells with a phosphospecific antibody. In LCL-N (Figure 27C), a phospho-Thr387 signal became detectable after 0.5Gy, but not 0.25Gy, and markedly increased with the dose of IR (up to 14 fold after 4Gy). By contrast, the Chk2-Thr387 phosphorylation was undetectable in irradiated AT and NBS cells (Figure 27C), concordant with the ATM- and Nbs1-dependence of this event.

As an additional indicator of Chk2 activity *in vivo,* I evaluated the CDK inhibitor $p21^{waf1}$, whose radiation-induced expression depends on Chk2 (Hirao *et al.*, 2000; Takai *et al.*, 2002). In LCL-N cells, $p21^{waf1}$ protein was markedly induced at 3hrs only in response to doses >1Gy, whereas in AT this induction was defective (Figure 28).

As Chk2 mediates DNA synthesis arrest in response to IR through the phosphorylation of Cdc25A (Falck *et al.*, 2001), we analysed the radiation dose-dependent occurrence of this event using radiolabelled thymidine. In LCL-N cells the RDS detected (Figure 28), while unaffected by doses of IR between 0.25 and 0.5Gy, was slightly inhibited by 1Gy but significantly by higher doses. Conversely, in AT cells, no DNA synthesis inhibition was seen at every dose tested, while in NBS cells a partial inhibition is detectable only at 4Gy IR (Figure 28) in accordance with the defective S-phase checkpoint typical of these syndromes, which should be almost in part ascribed to Chk2 impaired activation. Altogether, these findings demonstrate that Chk2 becomes activated only in response to a threshold number of DSBs, below which it remains inactive despite its phosphorylation on Thr68 by ATM.

3.5 Chk2 response to hydrogen peroxide

The relationship between the DNA damage spectra and Chk2 responsiveness was further examined in cells exposed to sublethal concentrations of H_2O_2, an oxidant which primarily generates SSBs (Birnboim and Sandhu, 1997; Guidarelli *et al.*, 1997; and Figure

1A). In LCL-N, AT and NBS cells exposed to 20μM H_2O_2, the electrophoretic mobility of Chk2 remained unchanged for up to 2hrs, relative to untreated cells (Figure 29A). Conversely, a time-dependent Chk2 mobility delay in response to 100μM H_2O_2 was seen in LCL-N, but not in AT or NBS cells (Figure 29A). The extent of this phosphorylation-related shift, abolished by phosphatase treatment (data not shown), was similar to that seen after treatment with 4Gy. No changes in the basal levels of Chk2 kinase activity *in vitro* were seen in LCL-N, AT or NBS cells after treatment with 20μM H_2O_2, whereas after treatment with 100μM H_2O_2 the kinase activity rose in LCL-N cells only (Figure 29B). To determine the effects of these treatments on ATM, its in vitro catalytic activity was examined in LCL-N cells. The results (Figure 29C) showed a slight increase in the basal levels of ATM activity in response to 20μM H_2O_2, and this activity increased further by treatment with 100μM H_2O_2. Regarding $p21^{wafl}$, induced levels of this protein were seen in LCL-N cells after treatment with 100μM, but not 20μM H_2O_2 (Figure 29D). The defective induction of $p21^{wafl}$ in AT cells (Figure 29D) underscored the ATM-dependence of this response. Together with the AET and γ-H2AX foci findings these results demonstrate a DNA lesion-dependent activation of Chk2, whatever the nature of genotoxic agent, e.g.: IR or oxidative stress. Furthermore, Chk2 appears not significantly responsive to SSBs and nucleotide base modifications.

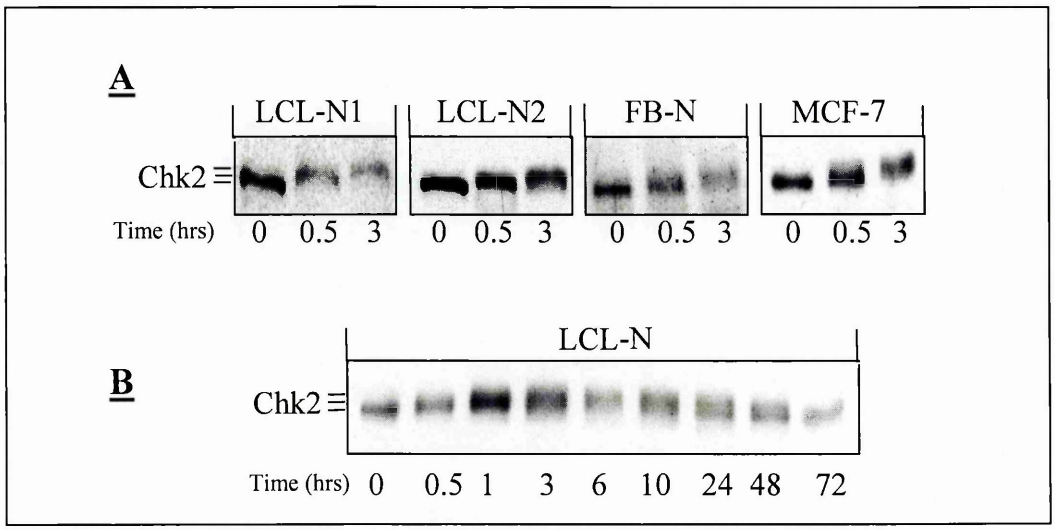

FIGURE 11: Time-course analysis of Chk2 phosphorylation following exposure to low dose γ-radiation. Western blots performed on exponentially growing LCL-N, LCL-N1, LCL-N2, FB-N normal cells and MCF7 breast cancer cells (**A**) and (**B**). Cells were harvested before or at the indicated time-points after 4 Gy of IR. Note the progressive increase in Chk2 retardation in the early hours post IR. In normal cells the mobility of Chk2 at 72hrs post IR is similar to that of untreated cells.

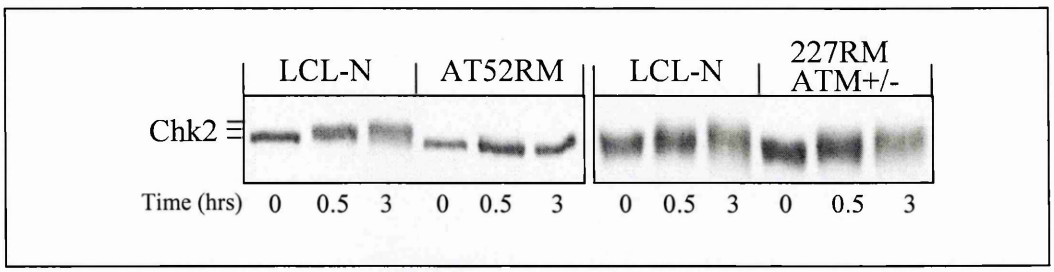

FIGURE 12: Chk2 phosphorylation in AT and heterozygous AT cells. Western blots performed on exponentially growing AT52RM (ATM -/-) and 227RM (ATM +/- cells). Cells were harvested before or at the indicated time-points after 4 Gy of IR. In both cell lines the mobility shift is similar to that of normal cells.

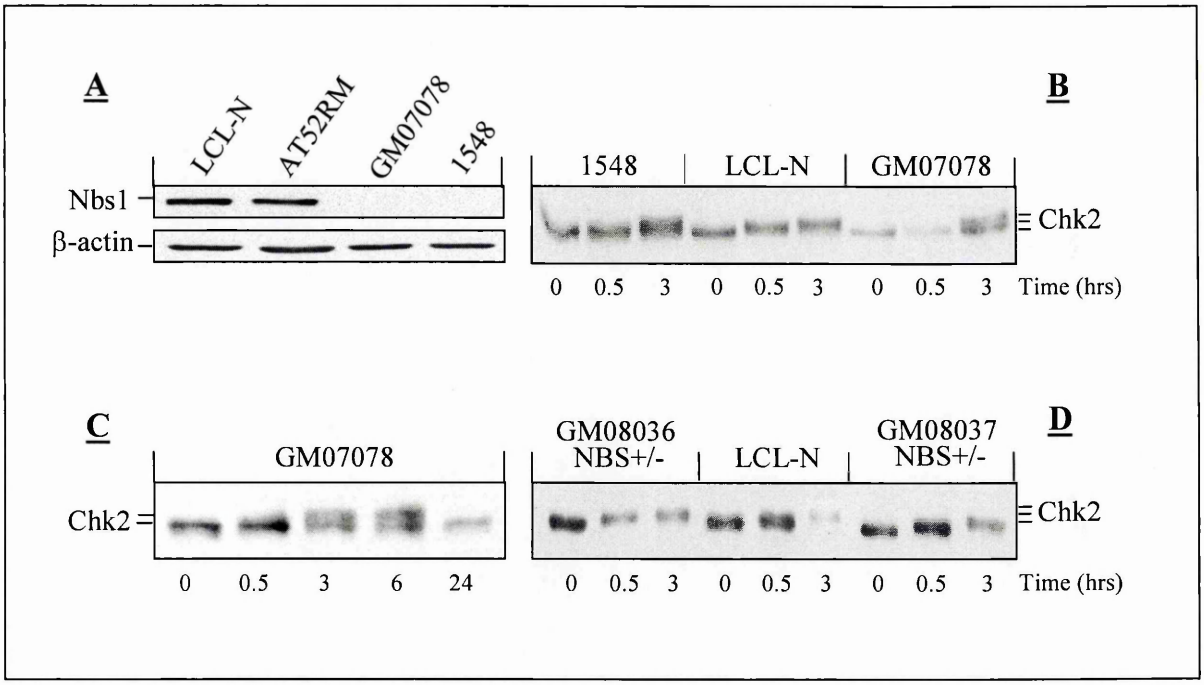

FIGURE 13: Nbs1 protein expression and time-course analysis of Chk2 phosphorylation in NBS cells. Western blots were performed on normal (LCL-N), AT (AT52RM), NBS (1548 and GM07078) and NBS-heterozygous (GM08036 and GM08037) cells harvested before or at the indicated time-points after 4Gy IR. In **A**, samples were tested for Nbs1 and afterwards for β-actin, to normalize lanes for protein content. Note the absence of Nbs1 protein in the two NBS cell lines. In **B**, **C** and **D**, samples were tested for Chk2. Note the absence of mobility shift 30min post IR in NBS cells, and normal Chk2 response in the NBS-heterozygous cells.

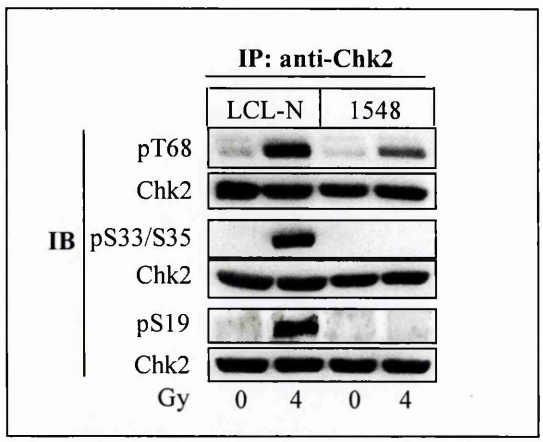

FIGURE 14: Chk2 phosphorylations in response to IR in normal and NBS cells. Phosphorylation at T68, S33/S35 and S19 were evaluated in LCL-N and NBS (1548) cell lines at 1 hr after IR treatment, using phosphospecific antibodies on immunoprecipitated Chk2 protein.

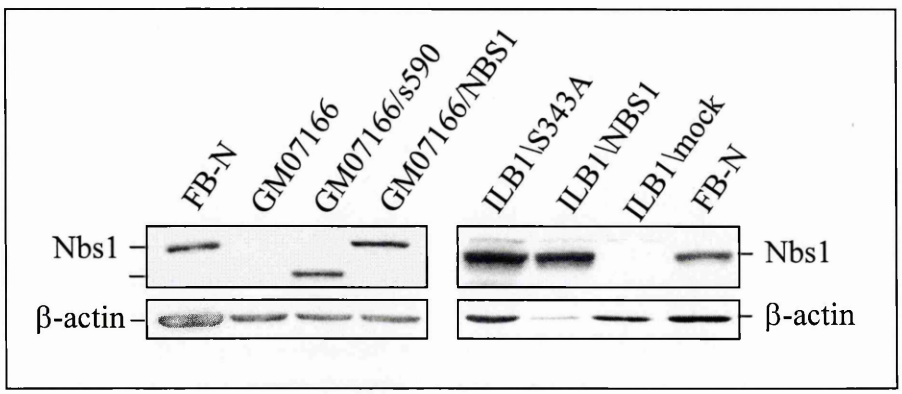

FIGURE 15: Nbs1 expression in NBS transfected cells. Lysates from the fibroblast cell lines GM07166/NBS1, ILB1/NBS1, GM07166/s590 and ILB1/S343A were western analyzed for Nbs1 to verify ectopic expression of the respective NBS1 cDNA constructs. Blots were reprobed for β-actin to normalize lanes for protein content.

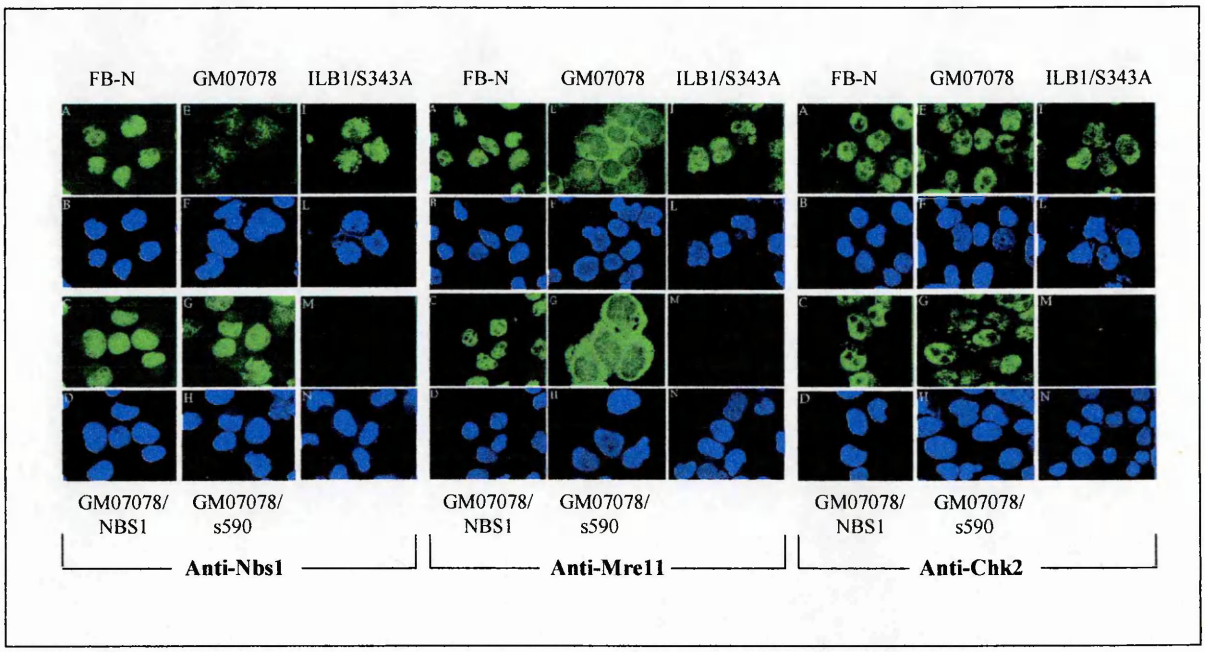

FIGURE 16: Nbs1, Mre11 and Chk2 localization in NBS transfected cells. Normal fibroblasts (**A-B**), NBS (**E-F**) and NBS cells stably expressing full length NBS1 (**C-D**), carboxy-truncated NBS1 (s590) (**G-H**), or S343A NBS1 (**I-L**). The negative control for each antibody, tested on normal fibroblasts is shown (**M**, **N**). Green and blue color images refer to immunofluorescence labelling and DAPI nuclear DNA staining, respectively.

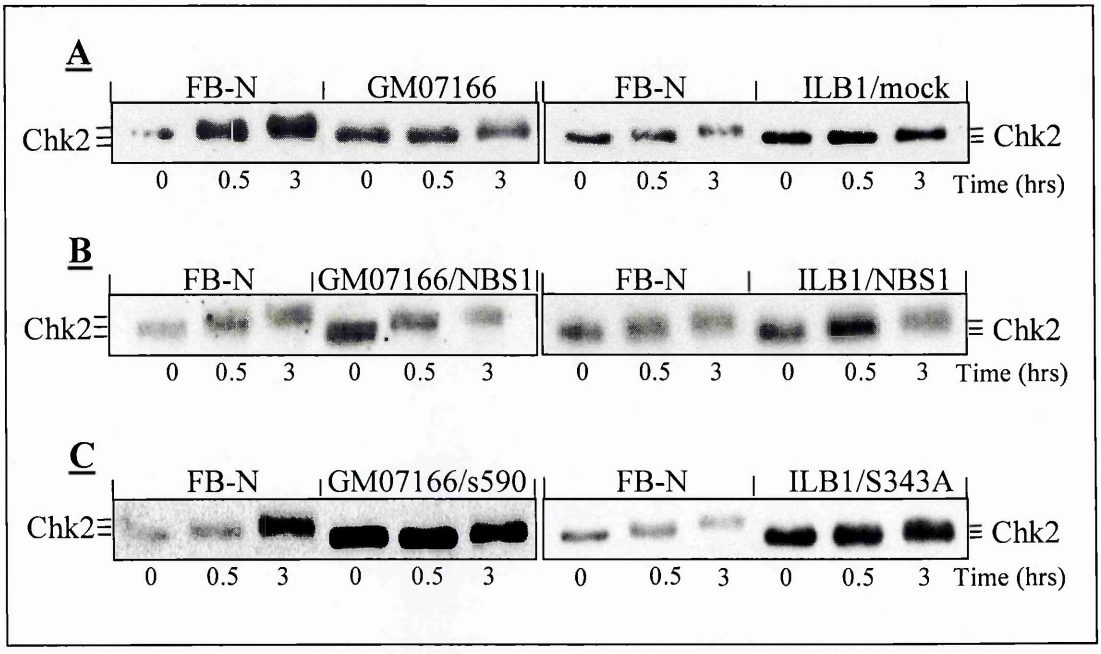

FIGURE 17: Chk2 mobility shifts in transfected NBS cells. Exponentially growing normal (FB-N) and NBS (GM07166, ILB1/mock) fibroblasts (**A**), NBS fibroblasts ectopically expressing either full length NBS1 cDNA (GM07166/NBS1, ILB1/NBS1) (**B**), a C-terminus deletion (GM07166/s590) or a S343A mutation (ILB1/S343A) (**C**), were harvested before or at the indicated time-points after 4Gy of IR and examined on western blots for Chk2.

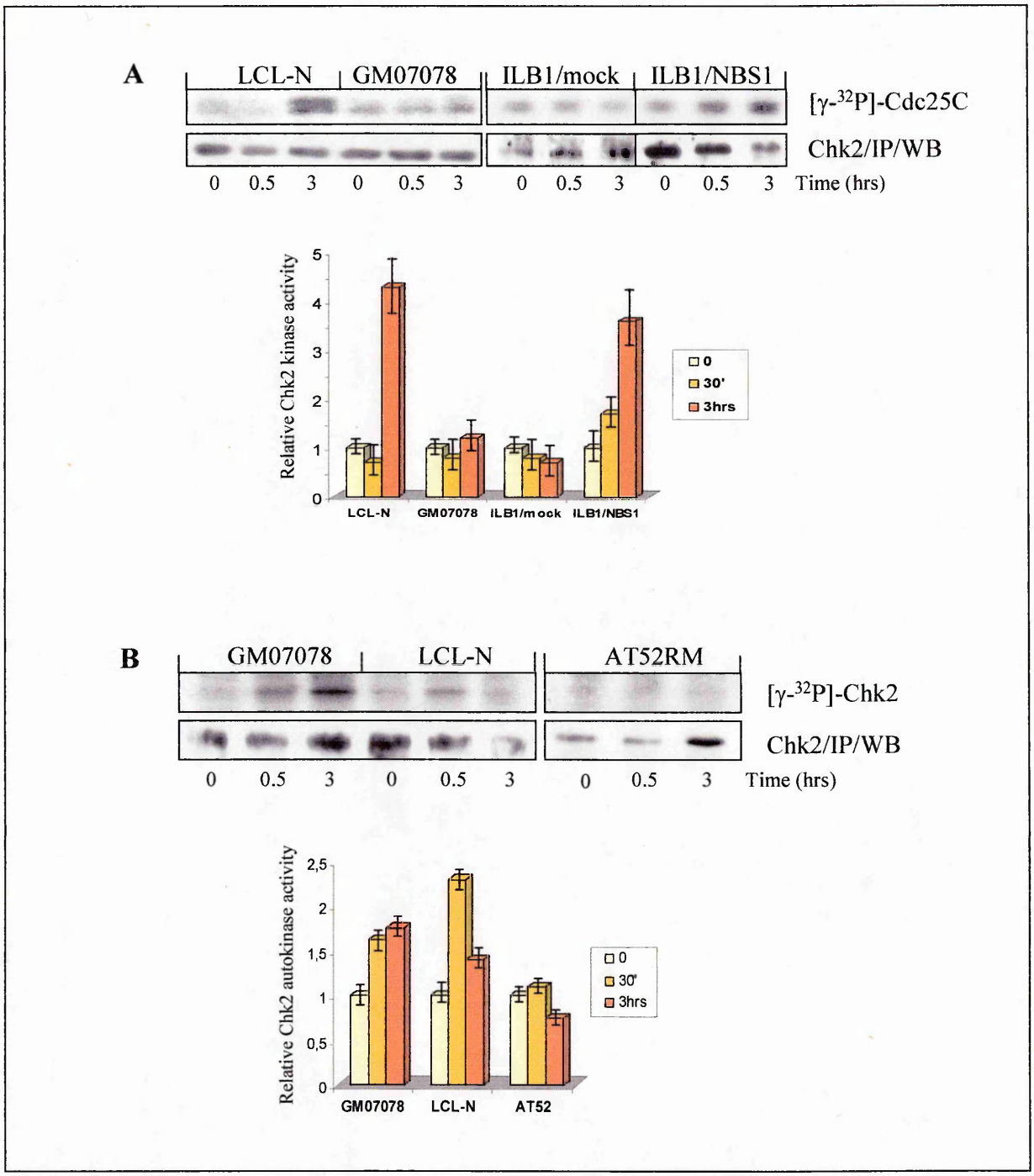

FIGURE 18: In vitro Chk2 kinase activity and autophosphorylation. Chk2 was immunoprecipitated from normal, NBS (GM07078, ILB1/mock) and NBS cells ectopically expressing wild type NBS1 (ILB1/NBS1) exposed or not to 4Gy γ-irradiation. Kinase reactions were assayed against a GST-Cdc25C recombinant substrate containing Ser216, the Chk2 target residue. The reactions were separated by gel electrophoresis and, after autoradiography, immunoblotted for Chk2 to verify that equal amounts of immunoprecipitated protein were present in each sample (**A**). Chk2 autophosphorylation was examined in kinase reactions in the absence of target substrate (**B**). The graphs were obtained by the densitometric analysis of autoradigraphic bands, as described in Methods, from three independent experiments. The reported kinase values were normalized for immunoprecipitated Chk2 content in each lane.

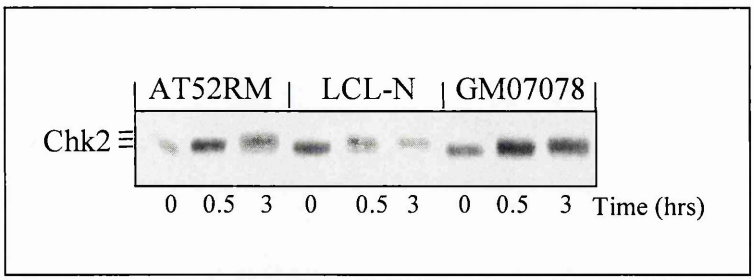

FIGURE 19: Chk2 mobility shifts in response to high doses of γ-radiation. Normal, AT and NBS cells were harvested before or after exposure to 50Gy of γ-radiation and western analysed for Chk2. Note the similar IR-induced Chk2 gel retardation in all cell lines, indicating an ATM- and NBS-independent Chk2 phosphorylation event.

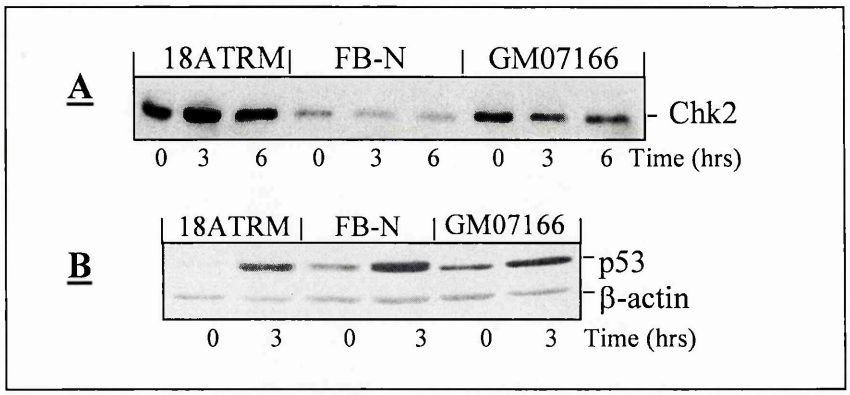

FIGURE 20: Chk2 mobility shift in response to UV irradiation. Normal, AT (18ATRM) and NBS (GM07116) fibroblast were irradiated with 10 J/m^2 and harvested at the indicated time points (**A**). Chk2 mobility shift was absent in all samples. To confirm the induction of a damage p53 accumulation was used as a reporter of a DNA damage response. P53 accumulate after the treatments in normal AT and NBS cell lines (**B**).

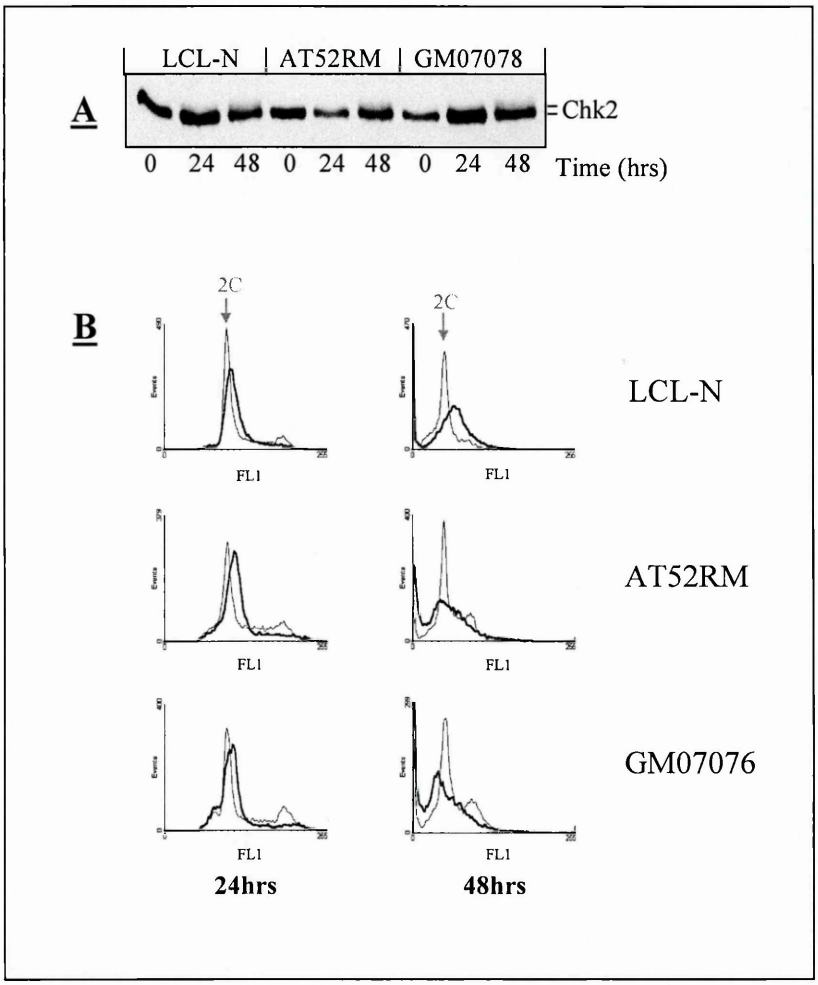

FIGURE 21: Chk2 regulation and cell cycle analysis after HU treatment. Cell, collected before or at the indicated times after exposure to 1mM HU, were split into two aliquotes, one probed on western blots with antibodies to Chk2 (**A**), the other analysed flow cytofluorimetry (**B**) to determine the S-phase arrest after treatment. Histograms in bold refer to HU treatments. The arrow indicates the position of the G1 phase peak. Horizontal and vertical axis display the relative fluorescence intensity and cell number, respectively.

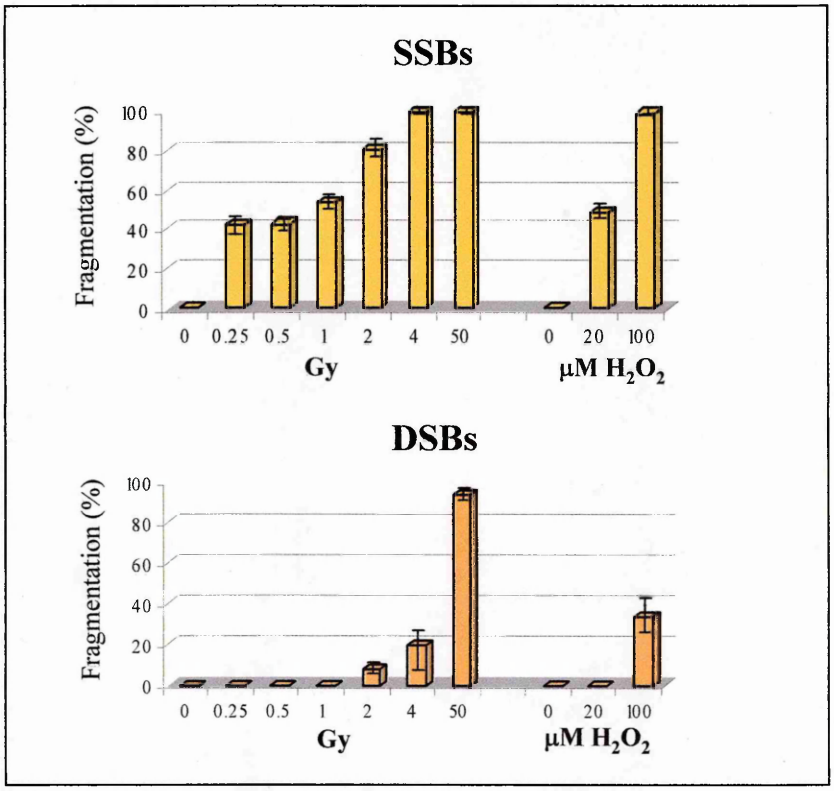

FIGURE 22: Assessment of DNA strand breaks by alkaline elution in response to γ-rad and H_2O_2. Immediately after irradiation on ice or 1hr after incubation with H_2O_2, the DNA from LCL-N cells was extracted and analysed by AET to quantitate SSBs and DSBs. The yield of these lesions is expressed as a fragmentation index, relative to untreated cells.

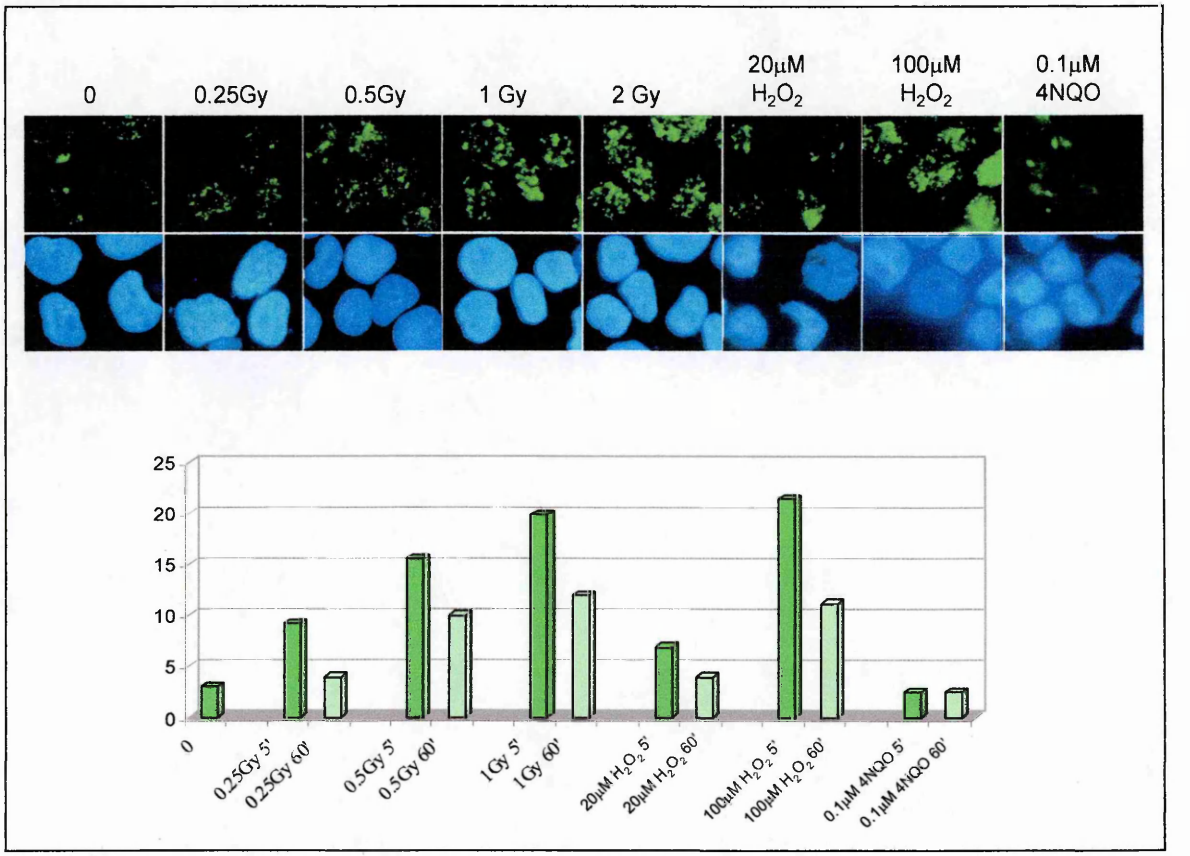

FIGURE 23: Quantification of γ-H2AX nuclear foci formation in response to γ-rad and H_2O_2. LCL-N cells were untreated (0) or treated with the indicated doses of γ-rad, 4NQO, H_2O_2 and permitted to recover for 5min or 1hr prior to harvesting, cytocentrifuged onto glass slides and labelled by indirect immunofluorescence with an anti-γ-H2AX antibody. Nuclear foci were counted by fluorescence microscopy.

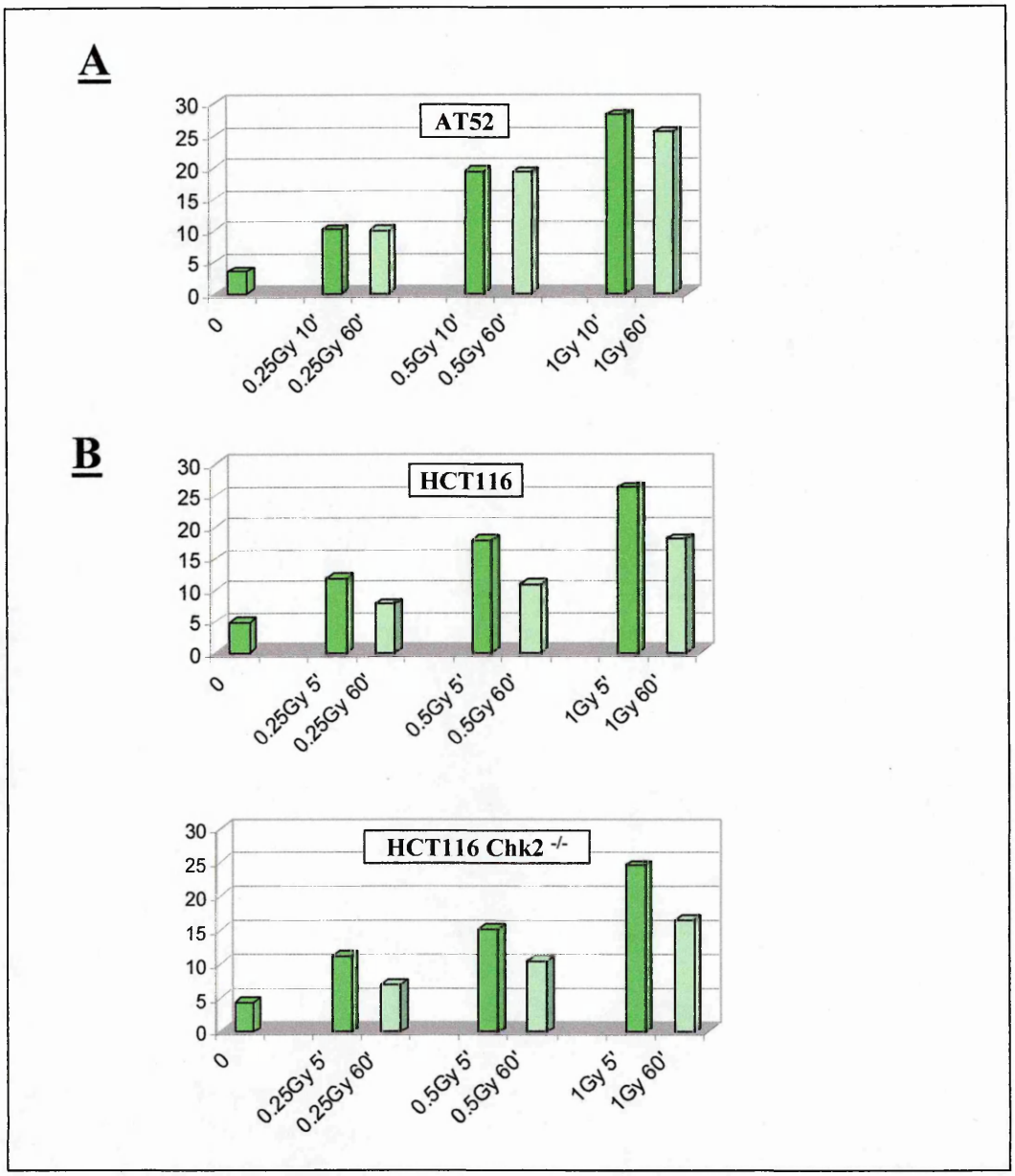

FIGURE 24: Quantitation of γ-H2AX foci in ATM-deficient, HCT116 and HCT116-Chk2$^{-/-}$ cells. AT52, HCT116 and HCT116 Chk2 KO cell lines were stained by immunofluorescence with the phosphospecific antibody and scored for γ-H2AX foci 5 min and 1hr post-IR. The data obtained in a representative experiment were represented in histograms.

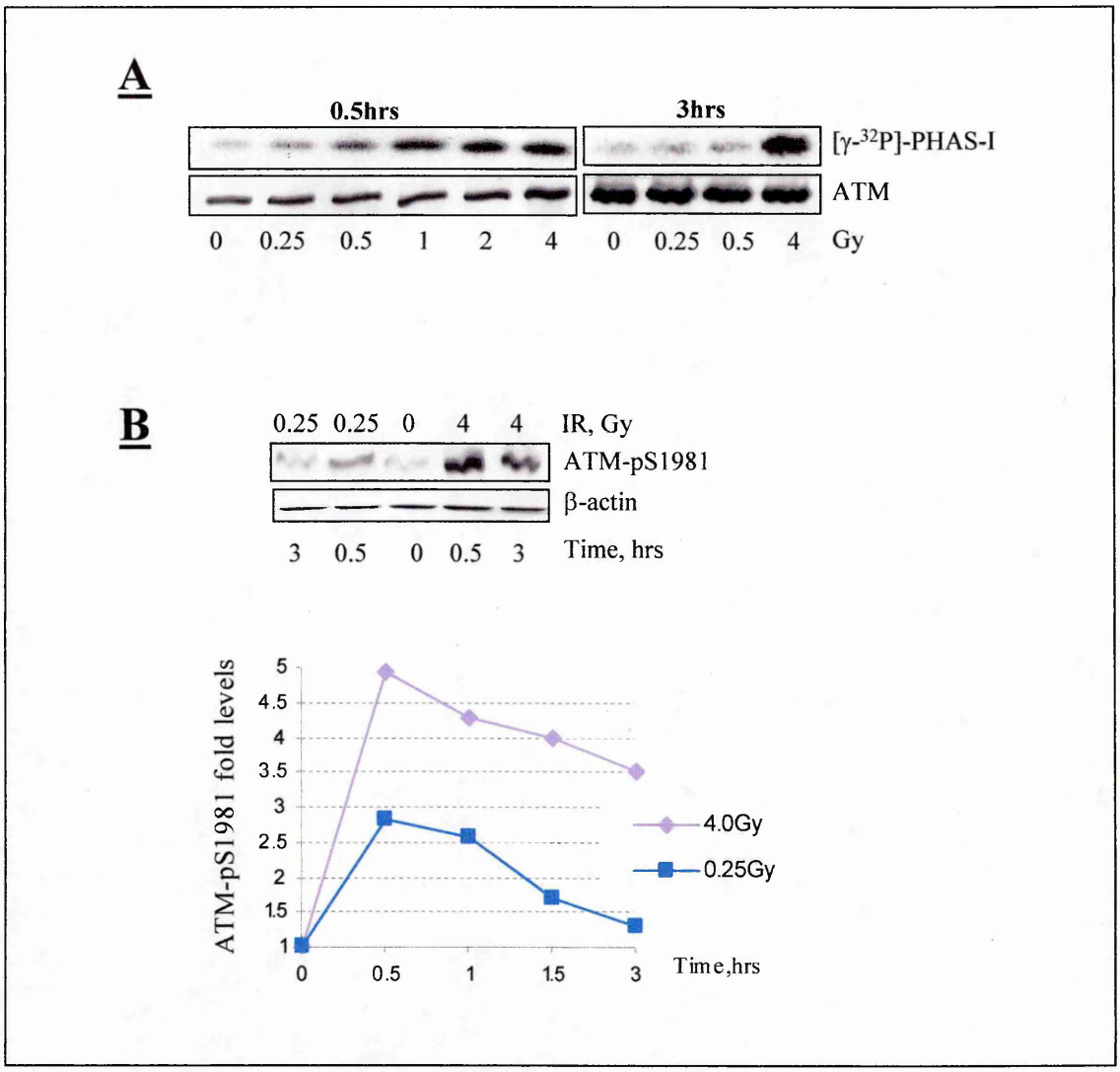

FIGURE 25: IR dose-dependent activation of ATM. The *in vitro* catalytic activity of ATM immunoprecipitated from LCL-N cells 30min and 3hrs (**A**) after irradiation was tested against the PHAS-I recombinant substrate. Following autoradiography, the reactions on gels were immunoblotted for ATM, to verify the amount of immunoprecipitated protein per sample. In (**B**), the time-dependent autophosphorylation of ATM-Ser1981 in LCL-N cells in response to low dose (0.25Gy) and high dose (4Gy) IR was examined by immunoblotting using a phosphospecific antibody. β-actin normalized the protein loading per lane. The histogram at the bottom was obtained from the densitometric analysis of ATM phospho-Ser1981 signals after normalization for β-actin (results are average of two independent experiments).

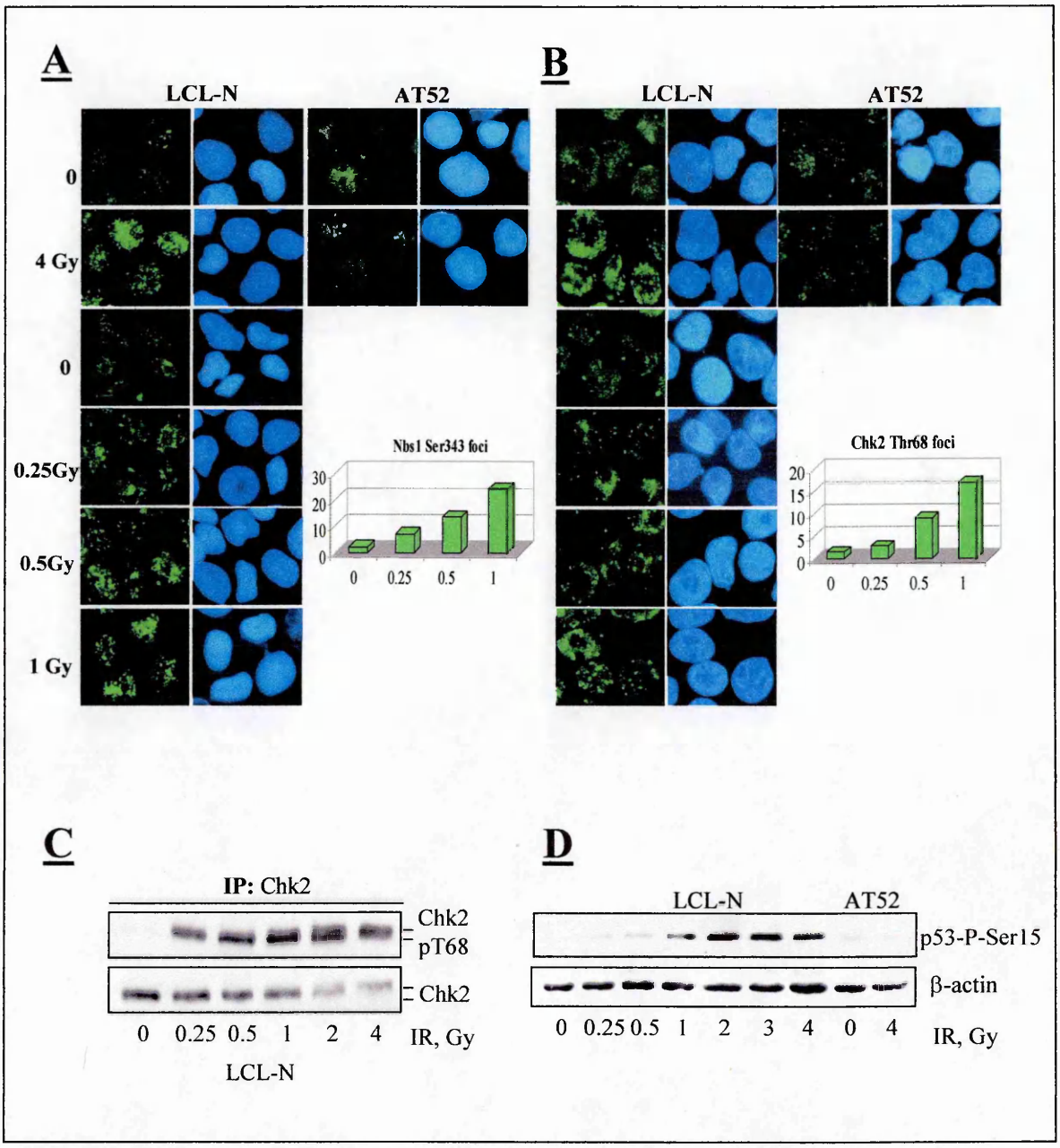

FIGURE 26: IR-induced phosphorylation of p53, Nbs1 and Chk2. Nbs1-Ser343 (**A**) and Chk2-Thr68 (**B**) phosphoresidues were detected by immunofluorescence staining with phosphospecific antibodies in LCL-N and AT cells before (0) and 30min after IR. The number of nuclear foci per cell in representative experiments is depicted in the histograms. The phosphorylation of Chk2-Thr68 (**C**) was detected on Chk2 immunoprecipitated from LCL-N cells 30min after irradiation by immunoblot with a phosphospecific antibody (top). Blots were reprobed with an anti-Chk2 antibody, to verify the amount of immunoprecipitated Chk2 per lane (bottom). The phosphorylation of p53-Ser15 phosphorylation was analysed by immunoblot (**D**) in LCL-N and AT cells 30min after irradiation. Reprobing for β-actin verified lanes for protein content.

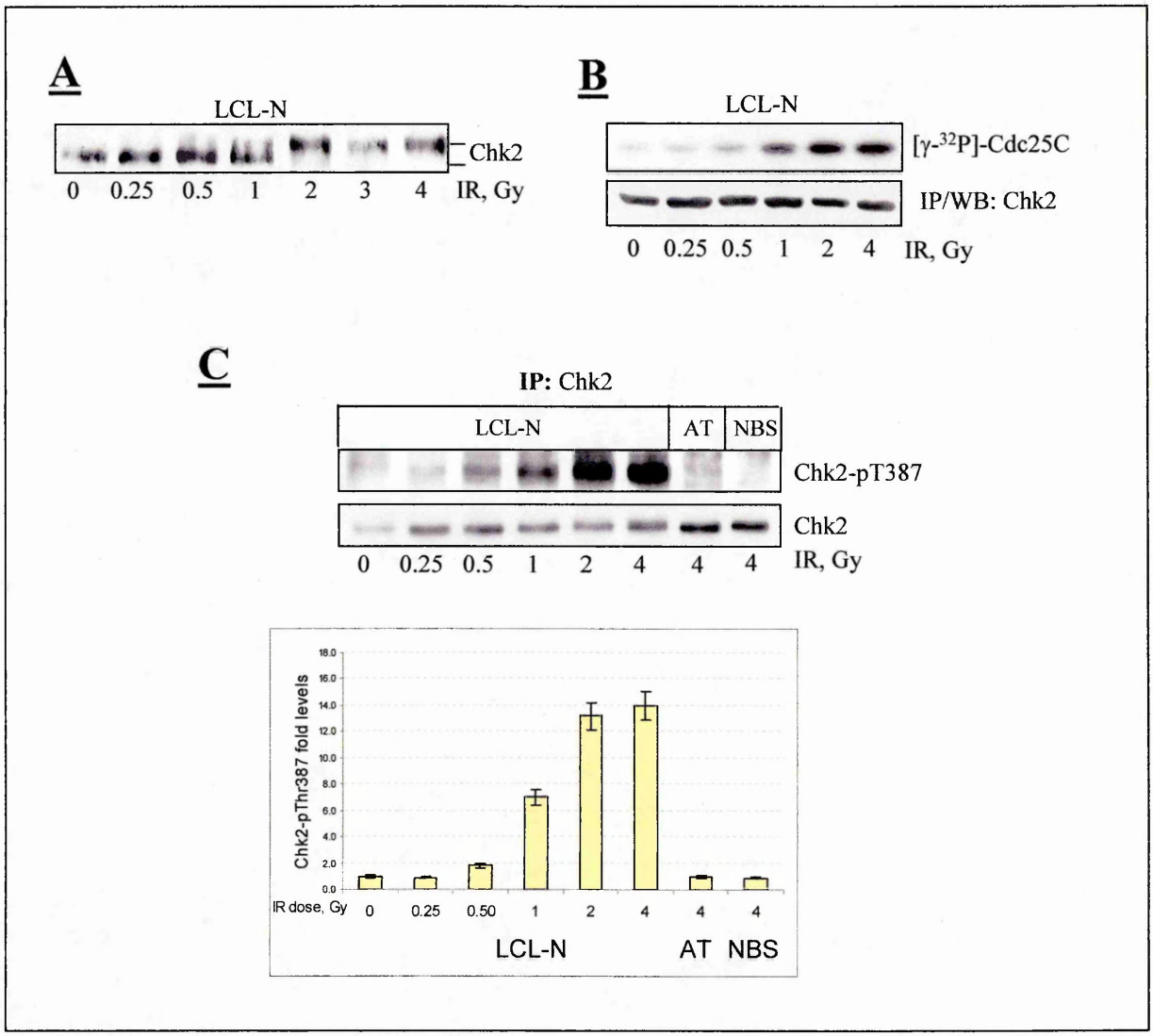

FIGURE 27: Chk2 mobility shift and catalytic activity after IR. Immunoblots for Chk2 (**A**) were performed on exponentially growing LCL-N cells 3hrs after exposure with escalating doses of IR. Note the progressive, phosphorylation-related upward shift of Chk2 becoming apparent after irradiation with doses above 1Gy. *In vitro* Chk2 kinase assays (**B**) were performed on the same cell preparations using a GST-Cdc25C recombinant substrate. The kinase reactions were resolved by gel electrophoresis and, after autoradiography, immunoblotted for Chk2 to verify the amount of immunoprecipitated protein per sample (Chk2 mobility shift is not detectable using this electrophoresis condition). The autophosphorylation of Chk2-Thr387 (**C**) was detected on western blot with a phosphospecific antibody on Chk2 immunoprecipitated from cells 45min after irradiation. The blot was reprobed with an anti-Chk2 to verify the amount of immunoprecipitated Chk2 per sample. The histogram below depicts the IR dose-dependent increase in Chk2-Thr387 phosphorylation, obtained by the densitometric analysis (+/- SD) of three independent IP/WB experiments.

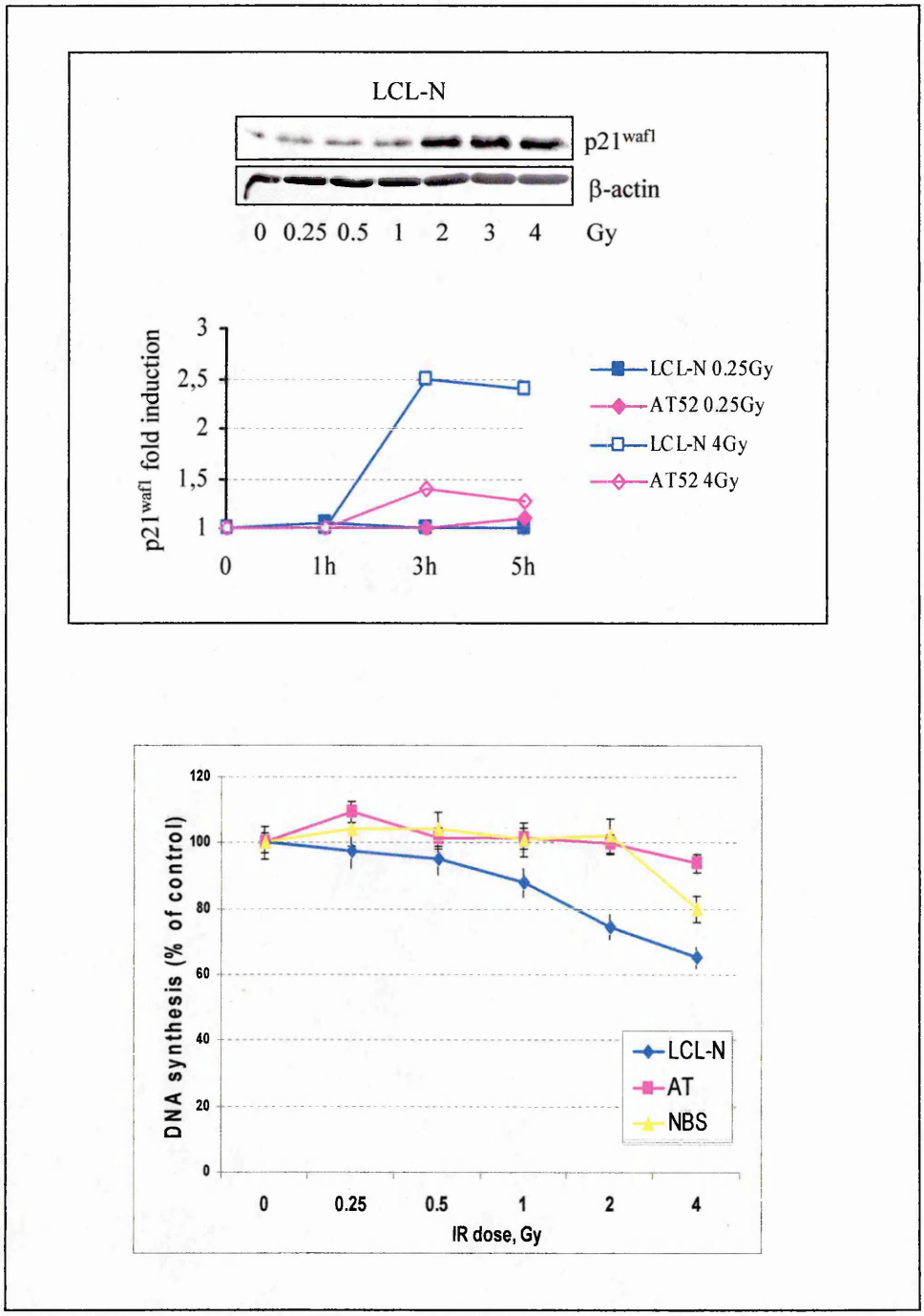

FIGURE 28: IR dose-dependent p21^{wafl} accumulation and radioresistant DNA synthesis. p21^{wafl} protein level in LCL-N cells 3hrs after treatment with the indicated doses of IR (**A, top**). Time-course analysis of p21^{wafl} protein accumulation in normal and AT cells after 0.25 and 4Gy (**A, bottom**). In (**C**), the RDS was evaluated on cells radiolabelled with [^{14}C] thymidine, irradiated and after 60min pulse labelled for 15min with [^{3}H]thymidine, as described in Materials & Methods. The ratios of [^{3}H] to [^{14}C] c.p.m. (corrected for c.p.m. resulting from channel crossover) were a measure of DNA synthesis. Data are average (+/-SD) of three independent experiments.

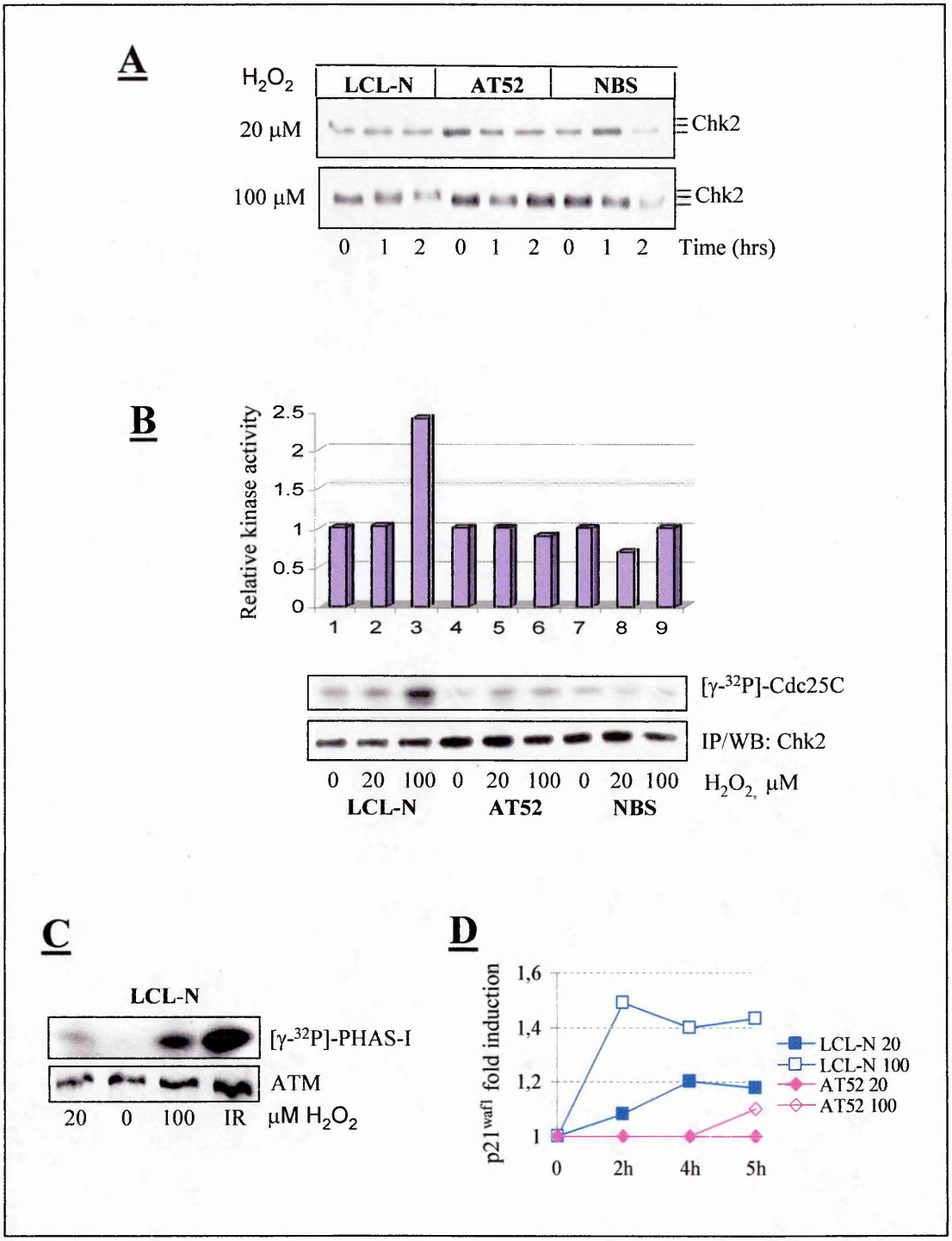

FIGURE 29: Chk2 mobility shift and activation after H_2O_2 treatments. Cells were harvested before, 1hr or 2hrs after incubation with H_2O_2. In (**A**) the immunoblot shows a phosphorylation-related upward shift of Chk2 only in normal LCL cells treated with 100µM H_2O_2. In (**B**), Chk2 was immunoprecipitated and tested for kinase activity *in vitro* (see legend to Fig. 2). The relative levels of enzymatic activity per lane, determined by densitometric analysis, are depicted. The effect of H_2O_2 on ATM activity (**C**) was examined in an *in vitro* kinase assays on ATM immunoprecipitated from LCL-N cells harvested 1hr after treatment with the oxidant. A lysate from irradiated cells was included as a positive control. In (**D**) time course analysis of $p21^{waf1}$ protein accumulation in normal and AT cells after 20 and 100µM H_2O_2.

DISCUSSION

Eukaryotic cells are under the constant attack of intrinsic and extrinsic genotoxic agents that potentially could induce damage to nuclear DNA. To avoid the catastrophic effects of these lesions on genomic patrimony cells evolved a complex network of pathways named "global DNA damage response" that arrests cells cycle progression to prevent DNA damage increase in number and complexity and to supply the time necessary to repair the lesions. Furthermore, in multicellular organism, in presence of extensive or unrepairable damage, cell is conducted to suicide (apoptosis) to prevent a cell from becoming cancerous and endangering the organism. The importance of the global DNA damage response for cells and organisms survival is underlined by the wide number and seriousness of syndromes caused by the impairment of this response and by the high incidence of cancer predisposition in the vast majority of these affections.

Mutations in ATM and NBS1 genes are responsible for Ataxia Telangiectasia and Nijmegen Breakage Syndrome, respectively, two disorders that share hypersensitivity to genotoxic agents, immunodeficiency and increased predisposition to cancer. ATM is a protein kinase which upon activation (Jeggo *et al.*, 1998) phosphorylates several target proteins (eg: p53, Chk2, Mdm2, RPA, BRCA1, c-Abl; Kastan and Lim, 2000) that are crucial for the regulation of checkpoint arrest and DNA repair (Lavin and Shiloh, 1997). Nbs1 is a component of the multifunctional protein complex containing Mre11 and Rad50, involved in recognition and processing of DNA lesions (Paull and Gellert, 1999; Petrini, 1999). Several lines of evidence suggested a functional link between these proteins, including the common G2 checkpoint defect exhibited by the associated diseases (Beamish *et al.*, 1996; Ito *et al.*, 1999), the DNA-damage induced phosphorylation of Nbs1 by ATM (Gatei *et al.*, 2000), and their physical association in the BASC super complex (Wang *et al.*, 2000).

The protein kinase Chk2 is phosphorylated and activated in response to IR (Matsuoka *et al.*, 2000), to further propagate the DNA damage signal along several pathways, causing cell-cycle arrest in the G1, S and G2/M phases; activation of DNA repair; and, in some cases, apoptosis (Falck *et al.*, 2001; Chehab *et al.*, 2000; Chaturvedi *et al.*, 1999; Zhang *et al.*, 2004; Chen *et al.*, 2005). Chk2 could participates to the G1/S arrest phosphorylating and regulating p53 and E2F-1 activation (Chehab *et al.*, 2000; Rogoff *et al.*, 2004) but also inducing Cdc25A degradation (Falck *et al.*, 2001), while could blocks G2/M progression excluding Cdc25C from nucleus (Chaturvedi *et al.*, 1999). Chk2 has been involved also in DSBs repair, a role carried out through the functional interaction with Brca1 (Zhang *et al.*, 2004), and, as suggested by $Chk2^{-/-}$ mice and mouse embryo fibroblasts (MEFs), in apoptotic activation (Zhang *et al.*, 2004).

The role of Chk2 as tumor suppressor was supposed for the involvement in the global checkpoint response and emphasized by the identification of germline and somatic mutations of the *CHK2* gene in human hereditary and sporadic tumours (for a review see Bartek and Lukas, 2003).

As Chk2 is activated by phosphorylation directly by ATM, and in consideration of a possible functional interaction between ATM and Nbs1, I have examined the ATM-dependent DNA damage response pathway in relation to the role of Nbs1 in the activation of Chk2, in the attempt to understand the molecular defects which characterize these two syndromes.

To finely describe Chk2 modification after damage and on the basis of the electrophoretic mobility changes on gels I have demonstrated a progressive time-dependent phosphorylation of Chk2 after IR, which becomes maximal between 1-3hrs and persists for up to 10hrs. Given that Chk2 contains seven putative major phosphorylation sites, considered responsible for electrophoretic mobility delay after IR (Matsuoka *et al.*, 1998; Melchionna *et al.*, 2000) these findings suggest that these residues are not simultaneously modified. Recently I have obtained data supporting this hypothesis by the analysis of five

phosphorylation sites which demonstrate different kinetics between *in trans* and autophosphorylation, the former occurring rapidly the second delayed in time after DNA damage (Buscemi *et al.*, 2006).

Chk2 phosphorylation after 4Gy IR was ATM and NBS1-dependent, as it was found defective both in cells derived from AT and NBS patients. It should be noticed, however, that in NBS cells Chk2 showed a minor mobility shift at 3hrs post IR, suggesting that at this time-point a limited and transient phosphorylation may occur in a fraction of the Chk2 pool of molecules. This partial change in Chk2 mobility could be explained by a low level of T68 phosphorylation, detectable in NBS cells. As in both normal and NBS cells Chk2 revealed the same nuclear immunostaining pattern, I can exclude a mislocalization phenomenon as the cause for defective Chk2 phosphorylation in Nbs1 deficient cells. I have also shown that decreased expression of ATM or Nbs1 protein does not impair the phosphorylation of Chk2, according to the analysis on AT- or NBS-heterozygous cells. In presence of >40% protein level both for ATM or Nbs1 a normal mobility shift is detectable in Chk2 protein. The normal behavior of AT-heterozygous cells is particularly intriguing since quite often other ATM-dependent events such as apoptosis or spindle checkpoint control, are compromised in these cells (Shigeta *et al.*, 1999). AT heterozygous are estimated about 1% in population and up to now it is not clearly defined if they are predisposed to cancer occurrence or to other aspects of ATM disease.

As Chk2 phosphorylation leads to its enzymatic activation (Matsuoka *et al.*, 1998), we assayed Chk2 kinase *in vitro* at different time points after IR. In these experiments, only the hyperphosphorylated form of Chk2 present in normal cells between 1-3hrs showed enhanced kinase activity towards Cdc25C substrate, whereas the phosphorylated forms of Chk2 present in normal cells at 30min, and in NBS cells at 3hrs, showed basal activity only. Therefore, the Chk2-induced response to DNA damage in NBS cells is, like AT, completely defective and only a fully phosphorylated form of Chk2 is active on substrates.

The presence of the same defect in two different NBS cell lines strongly suggest a direct relationship between impaired Chk2 activity and NBS1 deficiency, but to further provide evidence and to completely exclude the role of independent accumulating defects in NBS cell lines, I complemented these cells with a wild type copy of NBS1 gene. I demonstrated that in this cell line Chk2 phosphorylation and kinase activity are both restored after IR. Noteworthy, Chk2 phosphorylation was neither restored by an Nbs1 mutant protein truncated at amino acid 590, that deletes the Mre11-binding domain (Tauchi *et al.*, 2001), nor by a mutant in Ser343 (S434A), the ATM phosphorylation site on Nbs1. Mutants in Ser343 only partially complement S-phase checkpoint in NBS cells (Lim *et al.*, 2000). Immunofluorescence data demonstrated a diffuse Mre11 staining in cells transfected with truncated Nbs1 (s590), in contrast to the nuclear Mre11 staining of cells either normal or transfected with the wild type Nbs1, hence pointing out the essential function of the carboxy-portion of Nbs1 for the nuclear localization of the Mre11/Rad50 complex. Together with the other results on Chk2, these findings indicate that radiation-induced activation of Chk2 requires a correctly localized and phosphorylation-activated Nbs1-complex.

In *S. cerevisiae,* the activation of the Chk2-homologue Rad53 occurs by two interdependent steps, the first involving phosphorylation by Mec1 (the homolog of human ATM), the second an autophosphorylation process (Pelliccioli *et al.*, 1999). Evidences for the autophosphorylation of human Chk2 *in vitro* and *in vivo* by IR have been also provided (Lee and Chung, 2001). In this study, the IR-induced autophosphorylation of Chk2 *in vitro* was undetectable in AT compared to normal cells and partial in NBS cells. However in NBS cell lines at the time-point when Chk2 appeared modestly phosphorylated it was catalytic inactive against Cdc25C substrate (e.g.: at 30min and 3hrs post IR in normal and NBS cells, respectively). Hence, in the light of these findings, it is conceivable that in normal cells low doses of IR elicit an *in trans* phosphorylation of Chk2 by ATM and thereafter by other phosphorylation changes sustained by Nbs1. However we cannot

exclude the participation of another kinase that may potentially cooperates with ATM in Chk2 phosphorylation.

Other works suggested that Chk2 activation was ATM dependent only in presence of a limited amount of damage but became independent from ATM protein in presence of extensive damage, induced by very high doses of IR (Matsuoka *et al.*, 1998). To evaluate if, at these conditions, Chk2 activation became also Nbs1-independent we examined Chk2 phosphorylation status in AT and NBS cells exposed to high doses of γ-radiation (50Gy), demonstrating that it undergoes phosphorylation in a NBS- and ATM-independent manner. Whether this arises from the activity of ATR, an ATM-related protein kinase whose function partly overlaps with ATM, cannot be excluded. In this regard, it is worth noting that the closest human homologue of *S. pombe* Rad3, the modulator of the Chk2 homologue Cds1, is ATR (Bentley *et al.*, 1996). In any case, the finding that ATM and NBS1 are either involved or not in the activation of Chk2, depending on the extent of DNA damage, agrees with a cooperative role of Nbs1 and ATM in a DNA damage signal transduction pathway.

Collectively, my results support a model whereby the cooperation between ATM and NBS1 is an essential condition for the rapid and sustained activation of the checkpoint kinase Chk2 in response to low doses of IR DNA damage, thus providing an insight to better understand checkpoint defects in NBS cells. Recently some papers have shown a role for Nbs1 as an enhancer of ATM activation and an essential adapter for the kinase activity of ATM on substrates (Dupre *et al.*, 2006; Morales *et al.*, 2005; Difilippantonio *et al.*, 2005; You *et al.*, 2005; Paull and Lee, 2005), thus supporting the results described in this thesis. Furthermore I have demonstrated that a functional Nbs1 complex is essential for a correct phosphorylation by ATM of low affinity phosphorylation sites in Chk2 protein (Buscemi *et al.*, 2006).

Among the different types of DNA lesions generated by the normal metabolism or exposure to genotoxic agents, DSBs are extremely harmful as they can lead, if unrepaired,

to chromosomal breaks, rearrangements and tumor development (Khanna and Jackson, 2001).

While many underlying biochemical and functional features of Chk2 have been disclosed in recent years, the precise nature of the DNA lesions that evoke the phosphorylative activation of Chk2 remain unclear, if not controversial. Studies in *Xenopus* demonstrate the activation of *X*Chk2 by DNA oligonucleotides carrying double but not single strand ends (Guo *et al.*, 2000). Likewise, *C. elegans* Chk2 shows a key role in meiotic recombination (MacQueen and Villeneuve, 2001), a process involving the formation of DSB intermediates. However, human Chk2 seems responsive to UV or hydroxyurea (Brown *et al.*, 1999, Tominaga *et al.*, 1999), two agents that primarily cause pyrimidine dimers together with other bulky adducts and stalled replication forks, respectively, but that may induce DSBs at high doses and with extended treatments or as secondary events during the DNA repair process (Golos and Malec, 1991). In our hands the treatments of lymphoblastoid cells with 1mM HU for 18 hours or the exposure to 10 J/m^2 UV irradiation activate a DNA damage response in an ATM independent way but did not induce a Chk2 mobility shift comparable with IR treatment. As we have demonstrated that only a completely shifted Chk2 protein is active on substrates, these data seem to underline the role for Chk2 in DSBs response as also suggested by its ATM and Nbs1-dependent activation. AT and NBS cells, which carry defects in these proteins, are in fact hypersensitive specifically to DSB-inducing agents (e.g.: γ-rad) (Huo *et al.*, 1994; Kraakman-van der Zwet *et al.*, 1999). Moreover, AT and NBS patients show an immunodeficiency due to inappropriate resolution of DSBs associated with V(D)J and class switch recombination intermediates (Perkins *et al.*, 2002; Petersen *et al.*, 2001). While Chk2 activation in response to DSBs is a widely accepted event, the same activation after damaging agents like UV and HU seems more debated. Some papers have described a change in Chk2 mobility shift in response to UV or HU treatments (Matsuoka *et al.*, 1998; Brown *et al.*, 1999; Tominaga et al., 1999; Stiff *et al.*, 2006) although in any case only a

partial and delayed in time modification has been detected. This discrepancy in part could be explained by differences in cell lines (primary, immortalized, tumor cells) or in genotoxic agents exposure concentration and time. Another source of variability should be the phase of cell cycle when damage occurs: in fact Chk2 has been described as phosphorylated in response to UV treatments specifically in S/G2 phase of the cycle (Stiff *et al.*, 2006), while the cell lines used in this study were for 70-80% of the total population in G1 phase. The markers chosen to reveal Chk2 activation are another element of confusion. For example T68 phosphorylation or a partial mobility shift seems not indubitably correlate with a significant Chk2 kinase activity on substrates (Ahn and Prives, 2002). Particularly I found T68 phosphorylated in any case of DNA structure stress (Buscemi *et al.*, 2006) including after cells transfection, this event not leading to Chk2 activation or cell cycle arrest (data not shown). Recently I have also demonstrate the three ATM-dependent phosphorylation sites located in TQ/SQ region of Chk2 are not modified in response to UV or HU treatments (Buscemi *et al.*, 2006), thus confirming a limited role of Chk2 in the cellular response to these agents.

The data obtained using different damaging agents prompt us to investigate the amount and characteristics of damage sufficient to activate a specific checkpoint response. This is particularly interesting not only for a more precise description of a molecular mechanism but also for the widespread application of DNA damaging agents as chemo- and radiation therapy in cancer. This is more relevant when radiosensitive patients, as AT and NBS are, should be treated for tumors occurrence.

To better understand the lesion-specificity of the Nbs1-ATM-Chk2 pathway I have examined the relationship between the spectrum and yield of inflicted DNA lesions and the activity of ATM and Chk2 kinases, to determine the threshold amount of initial damage capable of triggering an ATM-Chk2-dependent cell cycle checkpoint response. For this purpose, the DNA was extracted immediately after exposure of cells to genotoxic agents and analyzed by AET to estimate the yield of SSBs and DSBs (Kohn *et al.*, 1981). This

approach is useful because it can discriminate SSBs from DSBs but, as other existing techniques for measuring genomic DNA strand breaks, i.e. comet assay and pulsed-field gel electrophoresis, underestimates DSBs yields, due to sensitivity limits and non-random distribution of generated breaks (Prise *et al.*, 2001). I therefore evaluated DSBs on the same samples also by the detection of γ-H2AX which rapidly accumulates (<5min) at the sites of DNA breaks to form foci (Rogakou *et al.*, 1999). Indeed in several studies, γ-H2AX foci staining have been used as a sensitive marker for DSBs (Rogakou *et al.*, 1999; Petersen *et al.*, 2001; Rothkamm and Löbrich, 2003; Furuta *et al.*, 2003; Jakob *et al.*, 2003), the number of early γ-H2AX foci well correlating with the number of DSBs inflicted.

By combining the results obtained by the AET with those derived from the assessment of γ-H2AX foci, I have precisely estimated SSBs and DSBs induced by various treatments. I have shown that exposure of normal cells to 0.25-4Gy γ-rad generates a dose-dependent increase in fragmented DNA, resulting primarily in SSBs at every dose tested. About 9 DSBs are induced by 0.25Gy IR and this number increase in a dose dependent manner.

As reactive oxygen species produced during mitochondrial respiration and peroxisomal metabolism are the major intracellular sources of DNA damage (Shackelford *et al.*, 2000), I analyzed the effect of increased intracellular free-radicals following treatment with H_2O_2, an oxidant which primarily generates SSBs (Birnboim and Sandhu, 1997; Guidarelli *et al.*, 1997). In LCL-N cells, treatment with 20 and 100μM H_2O_2 generated, besides an extended level of SSBs, amounts of DSBs comparable with 0.25Gy and 1-2Gy IR respectively.

To correlate DNA lesions yields and ATM-Chk2 pathway triggering I analyzed ATM and Chk2 activity in response to increasing doses of IR.

To thoroughly assess the regulation of ATM I utilized an *in vitro* kinase assay and I detected by immunoblot or immunofluorescence the phosphorylation of p53 Ser15, Nbs1 Ser343 and Chk2 Thr68, three well-established ATM targets (Shiloh, 2003). All the data obtained demonstrate that even a 0.25Gy exposure elicit in cells a rapid ATM respond, thus underlining the capacity of this kinase to sense and response to subtle DNA damages. Furthermore ATM induction is dose dependent and reaches a maximum at dose >2Gy, while at low doses (0.25-0.5Gy) the activity is unsustained up to 3hrs.

The ATM-dependent phosporylative activation of Chk2 leads to the phosphorylation of Cdc25A and Cdc25C, which are involved in S and G2-M phase checkpoints (Bartek and Lukas, 2003). Furthermore, $Chk2^{-/-}$ mouse cells also showed a significant impairment of $p21^{waf1}$ accumulation (Hirao *et al.*, 2000; Takai *et al.*, 2002), which may be attributable to a Chk2 activity on p53. Therefore collectively evaluating the *in vitro* and *in vivo* Chk2 kinase activity and the Chk2-dependent events, $p21^{waf1}$ accumulation and S-phase arrest, I have shown that Chk2 activation is clearly detectable only at doses >1Gy. Comparing data obtained form different approaches concur to define this threshold dose abrogating the specific sensitivity of each assay (i.e. antibody sensitivity).

Comparing these data with the DNA breaks evaluation at the same doses of IR I was able to demonstrate not only that Chk2 activity is unaffected by SSBs, that are present in large numbers after 0.25-0.5Gy, but also that a threshold level of DSBs is necessary to induce Chk2 kinase and Chk2 dependent downstream events. Furthermore our results reveal that despite the ATM-dependent phosphorylation of Chk2 in Thr68 (an essential activation site) occurs in response to γ-rad dose as low as 0.25Gy, Chk2 activity is elicited in response to greater amounts of damage, demonstrating that this event is necessary but not sufficient for the complete activation of Chk2. In this regard, it is notable that other phosphorylation and autophosphorylation steps occur *in vivo* before Chk2 activation (Schwarz *et al.*, 2003). Particularly I have recently demonstrated that three newly identified Chk2 phosphorylation sites are modified only after doses of IR >1Gy, thus

correlating with the data described above (Buscemi *et al.*, 2006). It is conceivable that some adaptors and/or other kinases more than ATM could contribute to Chk2 activation at higher doses of IR.

Whereas ATM is immediately activated by low number of DSBs (>9), Chk2 activation is delayed (>0.5hrs) and requires a higher number of DSBs (>15). This kind of response is consistent with a role for ATM in damage repair (Shiloh, 2003) and in a rapid cell cycle arrest (Bakkenist and Kastan, 2003). On the contrary, the characteristics of Chk2 induction seem more consistent with a role for this protein in checkpoint sustainment and apoptosis, as suggested by knock out mice (Jack *et al.*, 2002).

Furthermore the presence in normal proliferating cells of a constitutive number of DSBs (Fig. 2 and Rogakou *et al.*, 1999) suggests that cells can cope with limiting amounts of DSBs by silently repairing them without enforcing cell cycle checkpoint arrests (Leroy *et al.*, 2001). Therefore only DSBs resistant to a first wave of repair activity (Nunez *et al.*, 1995; Wang *et al.*, 2001) could function as an activating signal for Chk2 pathway. This hypothesis is sustained by the absence of Chk2 activation at doses of genotoxic agents, 0.25Gy and 20μM H_2O_2, producing a damage rapidly and completely repaired, and could also explain the role of the repair complex Nbs1/Mre11/Rad50 in Chk2 activation.

Regarding the response to SSBs, it is possible that they elicit an ATR/Chk1 and/or DNA-PK-dependent response (Plumb *et al.*, 1999). Furthermore, if misrepaired, these primary lesions could lead to secondary lesions, e.g.: during DNA replication when a fork passing through a template that contains a SSB converts the lesion into a DSB. This could explain the activation of a G2/M accumulation checkpoint detectable 24hrs after 0.25Gy (data not shown). On the contrary the analysis on normal cells showed a dose-dependent suppression of DNA replication, that became detectable in response to 1Gy, but not to lower doses (e.g.: 0.25-0.5Gy) which prevalently generate SSBs. These findings thus exclude a rapid and robust activation of the S-phase checkpoint by SSBs.

Neither the mobility shifts nor the catalytic induction of Chk2 was detected in normal cells exposed to 20μM H_2O_2, despite the marked presence of SSBs (and a low number of DSBs). However, treatment with 100μM H_2O_2, which generates a large amount of DSBs, elicits an ATM dependent response as evident by the catalytic activation of Chk2. These responses overlap those seen after 2-4Gy IR, treatments inducing similar yields of DSBs as 100μM H_2O_2. Altogether, these findings indicate that the Chk2 pathway is activated by DSBs, irrespective of the nature of the DNA damaging agent.

My work provides novel insight about the lesion and dose specificity of checkpoints triggering, revealing significant differences in ATM and Chk2 activation that could explain the different roles of this proteins in the DNA damage response. Furthermore the evidences obtained by the dose-response analysis for genotoxic agents may aid to enhance the efficacy of radiation and other DNA directed anticancer agents used to treat ATM and Chk2 defective tumours.

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Buscemi G, Carlessi L, Zannini L, Lisanti S, Fontanella E, Canevari S, Delia D (2006) DNA damage-induced cell cycle regulation and function of novel Chk2 phosphoresidues. *Molecular and Cellular Biology* 26: 7832-7845.